REGULATION OF THE VARIABLE DIFFUSION BARRIER TO OXYGEN IN NODULES OF SOYBEAN

By

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Since I discovered several years ago, that I was living in a world where nothing bears out in practice what it promises incipiently, I have troubled myself very little about theories. I am content with tentativeness from day to day.

Thomas Hardy, 1882

DEDICATION

To my parents: Thurmon Wiley Purcell and Clementine Lord Purcell.

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Abstract of Dissertation Presented to the Graduate School
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REGULATION OF THE VARIABLE DIFFUSION BARRIER TO OXYGEN IN NODULES OF SOYBEAN

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The permeability of soybean nodules to O_2 influences nodule respiration and thereby the energy and reductant available to fix N_2. Nodule permeability is responsive to environmental conditions which have important agronomic impact. However, there is no mechanistic understanding of how nodule permeability is controlled.

Modulation of nodule permeability was hypothesized to result from varying the amount of water filling intercellular spaces of the nodule cortex. Intercellular water would thereby function as a diffusion barrier to O_2. Changes in intercellular water content were hypothesized to result from water potential gradients developing between the symplast and apoplast in response to environmental signals.

To test this hypothesis, components of bulk nodule water potential were measured under different conditions affecting nodule permeability. There was no indication that components of bulk nodule water potential had a controlling influence on nodule permeability. However, the water release properties of nodules were favorable for the regulation of nodule permeability by an osmotic mechanism. The bulk modulus of elasticity for nodules indicated a high degree of elasticity, which would allow small changes in solute distribution to move relatively large amounts of water between the cortical symplast and apoplast.

Nodule ureide content increased in response to treatments which affected nodule permeability. Based on these results, two tentative conclusions were drawn. First, under conditions of sub-ambient rhizospheric O_2, ureides accumulated in the cortical symplast, withdrawing water from intercellular spaces, and increasing permeability. Second, under water deficit conditions ureides accumulated in the cortical apoplast and prevented both a decrease in intercellular water content and an increase in permeability.

Nodule ureide accumulation under water deficit conditions may have resulted from decreased phloem import of water and therefore the amount of water available to transport ureides out of the nodule in the xylem. Experiments were conducted to separate the effects of phloem water supply from phloem carbohydrate supply. Results were consistent with the hypothesis that continual water supply was needed to export ureides through the xylem and maintain permeability and that carbohydrates were needed to maintain dinitrogenase activity.

CHAPTER 1
INTRODUCTION

The availability of reduced nitrogen is a fundamental requirement of all living organisms; however, no eucaryotic life forms have evolved the capacity to reduce, or fix, N_2. This capacity is restricted to a few free-living procaryotes and symbiotic associations between procaryotes and eucaryotic organisms (Sprent and Sprent, 1990).

The bacterial enzyme responsible for N_2 fixation, dinitrogenase (E.C. 1.18.6.1), is composed of two proteins: dinitrogenase reductase and dinitrogenase (for review, see Burris, 1991 and references therein). Dinitrogenase reductase functions as an electron shuttle, accepting an electron from either ferredoxin or flavodoxin, depending upon the system. With the hydrolysis of 2 MgATP, dinitrogenase reductase transfers the electron to dinitrogenase. The dinitrogenase component contains an FeMo cofactor which binds N_2 and, following the accumulation of 6 electrons from dinitrogenase reductase, passes the electrons to N_2 forming NH_3. Associated with the reduction of N_2 is the concomitant reduction of 2 protons to H_2, requiring an additional 4 MgATP and 2 electrons. On a molar basis, the theoretical stoichiometry for the reduction of 1 N_2 and 2 H^+ is 16 MgATP and 8 electrons.

In addition to requiring large amounts of energy and reductant to fix N_2, dinitrogenase is inactivated by O_2, necessitating a near anaerobic environment for activity. The demands of an O_2-free environment that is also rich in energy and reductant is a challenge to aerobic organisms dependent upon oxidative phosphorylation for energy needs. In the legume-rhizobia symbiosis these constraints are met by isolating and protecting dinitrogenase from O_2 in the central region of the nodule.

Three main systems work coordinately in soybean nodules to provide the high energy/reductant and low O_2 environment necessary for N_2 fixation (Layzell and Hunt, 1990). First, a diffusion barrier in the outer region of the nodule restricts O_2 entry into the infected region. Second, a high respiratory rate and bacteroid terminal oxidases with a high affinity for O_2 (Bergersen and Turner, 1990) rapidly consume free O_2 to a low level. Third, the O_2-binding protein, leghemoglobin, binds O_2 at the periphery of infected cells and moves down its diffusion gradient to deliver O_2 to the symbiosome at a high flux rate but low free O_2 concentration (Appleby, 1984).

Physiological studies indicate that the permeability of the diffusion barrier to gases changes in response to environmental signals and controls dinitrogenase activity at the nodule and plant levels. In intact plants, decreasing the rhizosphere partial pressure of O_2 (pO_2) below ambient levels results in a rapid decrease in dinitrogenase activity (Criswell et al., 1976; Weisz and Sinclair, 1987b) while gradually increasing the pO_2 from 20 to 30 kPa increases dinitrogenase activity (Hunt et al., 1989). Extending O_2 depletion or enrichment experiments results in

a return to rates of dinitrogenase activity equivalent to that at ambient pO_2 within a few minutes for individual nodules (Hunt et al., 1989) and in 8 to 24 hours for intact plants (Criswell et al., 1976; Weisz et al., 1985; Weisz and Sinclair, 1987b). Nodule respiration also returns to ambient pO_2 rates following changes in the rhizosphere pO_2 (Hunt et al., 1987, 1989; Weisz and Sinclair, 1987a), indicating that the permeability of the nodule to O_2 diffusion is altered rather than nodule respiratory consumption.

Besides the effects of rhizosphere pO_2 , there are additional environmental factors which affect nodule permeability. Nodule permeability and dinitrogenase activity increase with increasing soil temperatures (Weisz and Sinclair, 1988b). Such changes account for diurnal variation in dinitrogenase activity commonly observed when soil temperature is not controlled. Dinitrogenase activity and nodule respiration are decreased when detached nodules are desiccated, and this inhibition can be alleviated temporarily by increasing the pO_2 surrounding nodules (Pankhurst and Sprent, 1976). Studies of intact plants exposed to water deficits have revealed similar effects of O_2 enrichment on dinitrogenase activity (Pankhurst and Sprent, 1976; Weisz et al., 1985). Also shading (Ryle et al., 1985), defoliation (Hartwig et al., 1987), or stem girdling (Walsh et al., 1987) decrease dinitrogenase activity, which can be restored temporarily by O_2 enrichment of the rhizosphere. The positive response of dinitrogenase activity to O_2 indicates that nodule permeability is limiting dinitrogenase activity rather than a limitation of carbohydrate per se. Nodule permeability also decreases after NO_3^- application (Minchin et al., 1989; Schuller et al., 1988) or following prolonged exposure to

C_2H_2 (Witty et al., 1984). The effect of these diverse treatments on nodule permeability and dinitrogenase activity indicates that nodule permeability is a key control point of N_2 fixation.

The effects of soil O_2 tension, drought, and soil NO_3^- availability on legume growth and N_2 fixation are fundamental agronomic concerns. Decreased soil O_2 tension could potentially limit N_2 fixation in fields with a high water table or during rapid microbial respiration of turned-under crop residue. Drought stress in soybean commonly occurs during the growing season and may result in a shortening of the seed-fill period and decreased yields (Muchow and Sinclair, 1986). The effect of NO_3^- on N_2 fixation is a management concern in situations of potential NO_3^- carry-over in crop rotation, green manuring with legumes, and legume intercropping (Streeter, 1988).

The capacity to regulate nodule permeability functions as a protective mechanism against O_2, preventing the inactivation of dinitrogenase. For example, if the permeability of the nodule was not variable, a decrease in the carbohydrate supply to the nodule would decrease nodule respiration and the consumption of O_2; this would lead to an increase in the internal O_2 concentration and inactivation of dinitrogenase. By decreasing nodule permeability under carbohydrate limited conditions, the internal increase in O_2 is avoided and dinitrogenase will remain functional until the carbohydrate status of the plant improves.

The location of the diffusion barrier appears to be in the cortex of the nodule based upon results from three different experimental approaches: (1)

anatomical examination (Dakora and Atkins, 1990; Frazer, 1942; Parsons and Day, 1990; Sprent, 1972), (2) microelectrode measurements of O_2 gradients within the nodule (Tjepkema and Yocum, 1974; Witty et al., 1987), and (3) modeling studies (Hunt et al., 1988; Sheehy and Webb, 1991; Sinclair and Goudriaan, 1981).

Anatomically, the nodule may be divided into three main zones: the outer cortex, the inner cortex and the infected region (Figure 1-1; Frazer, 1942; Sprent, 1972; Walsh et al., 1989b). Radial bands of proliferated tissue appearing on the surface of nodules are lenticels and increase aeration in the outer tissue. Lenticels are generally located above the vascular bundles. An outer layer of cells form the outer cortex, and cells in this region are composed of loosely arranged, thin-walled parenchyma cells which freely exchange gases with the surrounding environment. Just inside the outer cortex is a single, incomplete layer of sclerid cells, which is continuous with the root endodermis (Walsh et al., 1989b). Positioned between the sclerid layer and the centrally located bacteria-infected region is a 5 to 10 cell thick region composing the inner cortex. Within the inner cortex are the vascular bundles, which are enclosed within an endodermis. A mature vascular bundle is composed of a central core of xylem surrounded by phloem and three layers of pericycle cells (Walsh et al., 1989b). A one to two cell thick layer is found in the inner cortex, which is tightly spaced and lacks intercellular air spaces. It is this region of the inner cortex that is believed to be the O_2 diffusion barrier. Interior to the inner cortex is the infected region, consisting of both greatly enlarged infected cells and smaller uninfected cells (Newcomb et al., 1985) in an approximate 1 to 1 ratio (Parsons and Day, 1990).

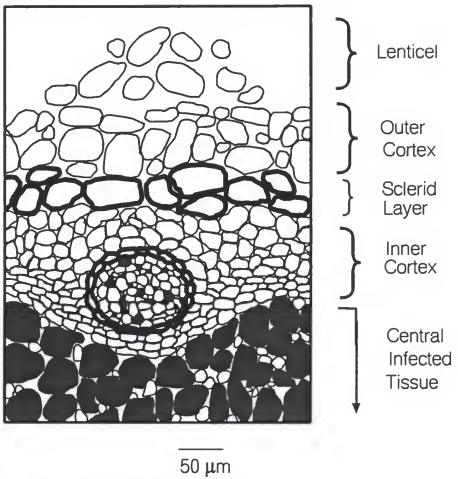

Figure 1-1. Schematic representation of a cross section from the outer region of a soybean nodule. Lenticels appear as radial bands of proliferated tissue on the nodule surface and generally lie above vascular bundles. Vascular bundles are found within the inner cortex and are surrounded by an endodermis. A one to two cell thick layer in the inner cortex is believed to function as the O_2 barrier. The central infected tissue is composed of enlarged infected cells (shaded) and smaller uninfected cells. Figure is based on photomicrographs from Parsons and Day (1990).

The abundance of intercellular air spaces in the infected region (Bergersen and Goodchild, 1973) allows unimpeded diffusion of O_2 within this region.

The anatomical features of a nodule described above are drastically altered by growing plants with their roots and nodules exposed to different O_2 concentrations. Low O_2 concentrations from 1 to 5% (v/v) cause a proliferation of lenticel development on the nodule surface of both cowpea (Dakora and Atkins, 1990) and soybean (Dakora and Atkins, 1991; Parsons and Day, 1990) and lead to an increase in the cortical intercellular air space content. Sub-ambient O_2 leads to both an increase in the thickness of the inner cortex and an alignment of the cell walls in adjacent cell layers (Dakora and Atkins, 1990, 1991). The alignment of cell walls in this region forms radial connections across the inner cortex and may allow for an increase in nodule permeability. Conversely, at ambient and supra-ambient O_2 concentrations, cells of the inner cortex are more elongated and walls of adjacent cell layers overlap. The overlapping of cell walls increases the tortuosity of the pathway to O_2 diffusion and may serve to decrease nodule permeability.

Microelectrode measurements of O_2 (Tjepkema and Yocum, 1974; Witty et al., 1987) and H_2 (Witty, 1991) gradients in nodules also indicate that the major site of resistance to gas exchange is the inner cortex. Progressive measurements of O_2 in the nodule from the outer surface to the infected region indicate that the outer cortex has a relatively high O_2 concentration and that there is a sharp drop in O_2 concentration across the inner cortex. The O_2 concentration in the infected region is uniform and extremely low. Conversely, H_2 specific microelectrode

measurements in soybean nodules infected with bradyrhizobia lacking an uptake hydrogenase have a relatively low H_2 concentration in the cortex that increases sharply as the electrode moves through the inner cortex into infected region (Witty, 1991). In this case, the inner cortex functions as a barrier to O_2 diffusion into the nodule, and as a barrier to H_2 diffusion out of the nodule.

Microelectrode measurements further indicate that the inner cortex is the site where the diffusion barrier is varied. When the O_2 microelectrode is passed just inside the inner cortex and the external pO_2 is increased from 21 to 40 kPa, there is a transient increase in the internal O_2 level which returns to the original pO_2 ambient level over a 2 to 4 minute period (Witty et al., 1987).

Modeling studies of gas diffusion into nodules have drawn together information from biochemical, physiological, anatomical, and microelectrode studies to provide a mathematical framework for examining gaseous transport (Hunt et al., 1988; Sheehy and Webb, 1991; Sinclair and Goudriaan, 1981). From these studies several important conclusions have been reached.

(1) Intercellular air pathways in the infected region are essential in providing a homogenous O_2 concentration. Without air pathways in the infected region, O_2 transport would depend upon diffusion in the liquid state and the nodule center would be anaerobic while O_2 concentrations at the edge of the infected region would be inhibitory to dinitrogenase.

(2) A diffusion barrier in the inner cortex is necessary to prevent O_2 inactivation of dinitrogenase. While the exact geometry of the

diffusion barrier is in question (Sheehy and Webb, 1991), a continuous shell of water surrounding the infected region from 20 to 40 μm in depth is adequate in providing the necessary resistance to O_2 diffusion. A barrier of this magnitude could be provided by 1 to 2 layers of tightly spaced cells (Hunt et al., 1988; Sinclair and Goudriaan, 1981).

(3) Leghemoglobin (Lb) is required in infected cells to facilitate diffusion of O_2 from the intercellular air spaces to the bacteroid (Hunt et al., 1988). There is only an estimated 6 nM O_2 gradient between the airspace at the surface of an infected cell and the bacteroid. Without Lb, the shallow O_2 gradient between intercellular air spaces and bacteroids would be insufficient to account for the calculated flux of O_2 required to give the observed rates of respiration (Layzell and Hunt, 1990).

A modeling approach has been used to account for the variability of the diffusion barrier (Weisz and Sinclair, 1988a). By assuming that the thickness of an aqueous shell surrounding the infected region changes from 10 to 70 μm, permeability of the nodule can be calculated for a hypothetical nodule by the relationship:

$$P_i = (D_i \bullet S_i) / L_x \qquad (1\text{-}1)$$

P_i, D_i, and S_i refer to the permeability, diffusivity, and solubility of gas "i" in an aqueous barrier, respectively, and L_x refers to the depth of the diffusion barrier. Permeability estimates for a particular nodule size can then be used to predict the

kinetics of C_2H_4 accumulation following the introduction of C_2H_2 assuming published K_m and V_{max} values of dinitrogenase for C_2H_2. While the model has been useful in quantifying the permeability response under different environmental conditions (Weisz and Sinclair, 1987b), a mechanism examining how the thickness of an aqueous diffusion barrier is regulated has not been explored.

One hypothesis for changes in the thickness of a water-filled diffusion barrier is that there are localized changes in the amount of water in cell walls and intercellular air spaces (Figure 1-2). Water movement in such a system is associated with water moving from an area of high potential to an area of low potential. Total water potential (Ψ_{tot}) of a cell may be divided into the sum of its component parts: the osmotic potential (Ψ_π), the turgor or pressure potential (Ψ_p), and the matrix potential (Ψ_m) (Kramer, 1983),

$$\Psi_{tot} = \Psi_\pi + \Psi_p + \Psi_m \qquad (1\text{-}2)$$

In most well-hydrated tissues, Ψ_m is small and may be omitted from the expression.

The volume flux density of water movement across a cell membrane (J_v) is proportional to the difference in water potential ($\Delta\Psi_{tot}$) inside and outside the cell (Nobel, 1991).

$$J_v = L_p (\Delta\Psi_{tot}) \qquad (1\text{-}3)$$

The proportionality coefficient, L_p, is the hydraulic conductance of the membrane. With the inclusion of the membrane reflection coefficient (σ) and the omission of Ψ_m, equations 1-2 and 1-3 may be combined (Nobel, 1991).

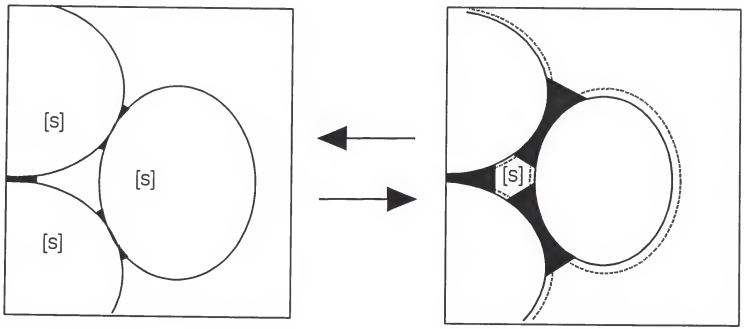

Figure 1-2. Schematic representation of proposed water flux in cells of the nodule inner cortex which may account for nodule permeability changes. An increase in intercellular water (shaded region) would decrease nodule permeability. Water movement between the symplast and apoplast is in the direction of decreasing water potential. Water potential gradients may be generated by changes in the solute [s] distribution between the affected cells and the intercellular spaces.

$$J_v = L_p (\Delta\Psi_p + \sigma\Delta\Psi_\pi) \quad (1\text{-}4)$$

In equation 1-4, $\Delta\Psi_p$ and $\Delta\Psi_\pi$ are the difference in pressure and osmotic potentials across a cell membrane, respectively, and σ describes the "leakiness" of the membrane to particular solutes.

Changes in Ψ_p, in response to water movement across a cell membrane, are dependent upon the elastic modulus (ϵ) of the cell and the relative change in cell volume ($\Delta v/v$) (Raschke, 1979):

$$\Delta\Psi_p = \Delta v/v \bullet \epsilon \quad (1\text{-}5)$$

Equation 1-5 may be included into equation 1-4 to describe water flux across a membrane:

$$J_v = L_p (\Delta v/v \bullet \epsilon + \sigma\Delta\Psi_\pi) \quad (1\text{-}6)$$

Regulation of water flux in a tissue in response to environmental signals will depend upon changes in the components of equation 1-6. The hydraulic conductivity, L_p, of pea stem tissue is constant under a wide range of treatments which affect water flux and tissue growth (Cosgrove and Cleland, 1983a, 1983b). Assuming that L_p is also constant in nodules, J_v will be controlled by ϵ, $\Delta v/v$, σ, and/or $\Delta\Psi_\pi$ (equation 1-6). The elastic modulus, ϵ, is determined by cell wall mechanics and interactions with cell volume, cell shape, and turgor pressure (Cosgrove, 1988). Elastic properties of a cell are crucial in determining the amount of solute required for a given change in cell volume (Raschke, 1979). However, if changes in ϵ do occur, they may be a response to, rather than a cause of, water flux into a cell. Similarly, changes in relative volume may reflect the influx or eflux of water rather than the cause of water movement. The reflection

coefficient, σ, affects water flux by increasing or decreasing leakiness for a particular solute. Changes in σ would lead to a redistribution of solutes across a membrane (i.e., $\Delta\Psi_\pi$) and a change in water flux. Guard cell movement is an example where σ changes in response to the environment by opening or closing potassium channels (Serrano and Zeiger, 1989). Similarly, water flux in the cells of pea stems is due to shifts in the apoplastic/symplastic concentration of solutes rather than changes in the hydraulic conductivity or the elastic modulus (Cosgrove and Cleland, 1983a, 1983b). An alternative explanation for changes in water flux is that instead of the reflection coefficient changing, the concentration of a particular solute changes in response to an environmental signal to affect $\Delta\Psi_\pi$.

The central hypothesis of this dissertation is that permeability changes are achieved through alterations in nodule water potential, which in turn are associated with changes in solute concentration and distribution. This hypothesis is simple and agrees well with the models of water movement control in stomata (Serrano and Zeiger, 1989), leaf pulvini (Campbell et al., 1981), and pea stems (Cosgrove and Cleland, 1983a, 1983b). However, the hypothesis has been a particularly challenging problem to approach experimentally in nodules due to four main factors: (1) the sheer number of compounds in nodules which could reasonably act as osmotica; (2) determining if changes in a proposed solute concentration occur in the cortex or the infected region; (3) determining how much solute is required to cause the necessary movement of water; (4) determining if there is an absolute change in solute concentration in the bulk nodule or if there is simply a change in the distribution of solute from the

apoplast to the symplast. The hypothesis of nodule permeability changes and the experimental limitations to these studies are examined in the following four chapters.

Chapter 2 examines the hypothesis that permeability shifts result in changes in the amount of water occupying intercellular spaces. Bulk nodule density and fractional nodule air space content were quantified in nodules differing in permeability. Bulk nodule Ψ_π was also measured to determine if permeability changes were associated with shifts in nodule solutes.

In Chapter 3, a pressure-volume curve was established for the bulk nodule in order to determine the elasticity of the tissue. Nodule elasticity determines the amount of solute required to give changes in nodule turgor. Soluble sugars, free amino acids, and ureides were also quantified from nodules differing in permeability properties to assess their potential role in regulating permeability.

Chapter 4 examines the hypothesis that permeability changes result from varying components of nodule water potential. However, instead of altering permeability and attempting to identify changes in Ψ_π (Chapter 2), nodule water potential was altered by manipulating the water status of the plant. The effect of these treatments on dinitrogenase activity, permeability, nodule respiration, nodule carbohydrates, and nodule ureides was monitored and compared to changes in nodule water potential.

In Chapter 5, the effect of altering the phloem supply on permeability and dinitrogenase activity was examined. Removing phloem supply by stem girdling (Walsh et al., 1987) or detaching nodules (Ralston and Imsande, 1982; Sung et al.,

1991) results in a rapid decrease in both permeability and dinitrogenase activity. This response has been attributed to a loss of cellular turgor in lenticels, resulting in a decrease in lenticel aperture and permeability (Ralston and Imsande, 1982) and to a decrease in carbohydrate supply provided by the phloem (Vessey et al., 1988). An alternative explanation for the effect of phloem supply on dinitrogenase activity and permeability is that a continual phloem supply is needed to provide the nodule with water, which functions as a solvent for the export of ureides through the xylem (Walsh et al., 1989a). An accumulation of ureides in the cortical apoplast would increase the thickness of a water-filled diffusion barrier and decrease dinitrogenase activity and permeability. Experiments in Chapter 5 describe providing water to nodules through the phloem in recently detopped plants and comparing changes in dinitrogenase activity and permeability over time with detopped plants without a source of phloem derived water.

CHAPTER 2
NODULE DENSITY AND OSMOTIC POTENTIAL RESPONSE TO CONDITIONS AFFECTING NODULE PERMEABILITY

Introduction

The flux density of O_2 into soybean nodules is decreased by the presence of a water-filled region in the nodule cortex (Hunt et al., 1988; Sinclair and Goudrian, 1981; Tjepkema and Yocum, 1974). The water-filled region functions as a diffusion barrier and maintains the O_2 partial pressure (pO_2) in the nodule interior at extremely low levels, preventing O_2 inactivation of dinitrogenase (Layzell and Hunt, 1990).

The flux density of O_2 (J, $mm^3\ mm^{-2}\ s^{-1}$) into nodules can be described by Fick's first law (Weisz and Sinclair, 1988a):

$$J = P\ (O_e - O_i) \qquad (2\text{-}1)$$

where P is the permeability ($mm\ s^{-1}$) of the nodule to O_2, O_e is the oxygen concentration external to the diffusion barrier ($mm^3\ mm^{-3}$), and O_i is the O_2 concentration inside the diffusion barrier ($mm^3\ mm^{-3}$). Since O_i is four orders of magnitude less than O_e (Layzell and Hunt, 1990), J is proportional to both O_e and P.

The effective thickness of the diffusion barrier is inversely related to P (equation 1-1). Changes in P are associated with specific environmental conditions which alter the diffusion barrier thickness. Sub-ambient pO_2's surrounding roots result in P increases, and supra-ambient pO_2's surrounding roots result in P decreases (Weisz and Sinclair, 1987b). Shading also lowers P (Ryle et al., 1985; Walsh et al., 1987) and prevents O_2 inactivation of dinitrogenase under conditions where respiratory consumption of O_2 is decreased due to carbohydrate limitation. Additionally, dinitrogenase activity is decreased by approximately 50% within 5 minutes of nodule detachment (Sung et al., 1991), and this response appears to be related to a rapid decrease in P.

Regulation of the diffusion barrier thickness may be due to changes in the amount of water in intercellular pathways of the nodule cortex. Variation of the intercellular water content associated with P changes could alter nodule density and the fractional air space content. Increases in P should increase intercellular air space content and decrease bulk nodule density. Conversely, decreases in P should decrease both the air space content and the bulk nodule density.

Variation in intercellular water content associated with P regulation is proposed to be due to changes in the water potential gradient between the symplast and apoplast (equation 1-3, Figure 1-2). For example, a decrease in the amount of osmoticant in a cell would increase cellular osmotic potential (Ψ_π). These conditions would lead to an efflux of water from cells and block intercellular pathways, decreasing P.

There were two objectives of experiments in this chapter. The first objective was to quantify bulk nodule density ($\bar{\rho}$) and the fractional air space content of nodules under conditions affecting P. The second objective was to compare bulk nodule osmotic potential ($\bar{\Psi}_{\pi}$) in intact plants following exposure of nodulated roots to different pO_2's.

Materials and Methods

Detached Nodule Experiments

Soybean seedlings (cv. Biloxi) were removed from an artificial soil medium (Terr-lite, WR Grace Co., Cambridge, MA) 3 to 4 days after sowing, and the root system was passed through a hole in a #3 rubber stopper. The plant-stopper combination was placed in the lid of a 38-L aquarium filled with half-strength, N-free Hoagland's solution (Hoagland and Arnon, 1938) and inoculated with a commercial preparation of Bradyrhizobium japonicum (Nitragin Co., Milwaukee, WI). The aquarium system was constructed to hold 6 to 8 plants, and each aquarium was continuously aerated with air (79:21, N_2:O_2) at a flow rate of 2 L min^{-1}. Aquariums were placed inside a thermally insulated chamber to modulate root and nodule temperature. Plants were grown in the greenhouse under natural illumination with the photoperiod extended to 16 h with incandescent lamps.

A plant with three to four fully expanded leaves was brought to the laboratory 2 days prior to conducting an experiment. The light source for plants in the laboratory was a combination of sodium and metal halide lamps delivering 2300 μmol m^{-2} s^{-1} PPFD at the top of the plant. The photoperiod was set for 16 h beginning at 0600 h.

Nodules greater than 2 mm in diameter were removed from the top 10 cm of one or two plants, and the nodules were pooled and randomly divided into two groups. The two groups of nodules were placed in separate test tubes, submerged in deionized water, and aerated at a flow rate of 200 ml min^{-1} with either a 21 kPa or a 10 kPa O_2 mixture. The length of the aeration treatment was 5, 60, 120, or 240 minutes. Each O_2 treatment and time combination was replicated 4 times.

At the end of aeration treatment, bulk nodule density ($\bar{\rho}$, g cm^{-3}) was determined for nodules of each pO_2 treatment by dividing the fresh weight (FW, g) of the nodule by nodule volume (v, cm^{-3}). Nodule volume was measured by Archimedes principle (Beiser, 1973) using the apparatus illustrated in Figure 2-1. A brass scaffolding was placed directly on the platform of an analytical balance. A plexiglass bridge supported a 250-ml beaker of water over the scaffolding and balance platform. A chamber, with the ends covered by 1mm x 1mm metal screen, was suspended from a small hook on the scaffolding by a nylon line into the beaker of water. The line and chamber assembly was easily detached from the scaffolding, and the chamber was composed of two separate pieces which screwed together.

To determine nodule volume, nodules were blotted dry and FW determined. Nodules were placed inside the chamber and immersed in the beaker of deionized water. The empty chamber had previously been tared while immersed in deionized water. Any change in weight of the chamber with nodules inside was due to the buoyant force (BF, g cm s^{-2}) of the nodules. Buoyant force of the nodules was calculated as:

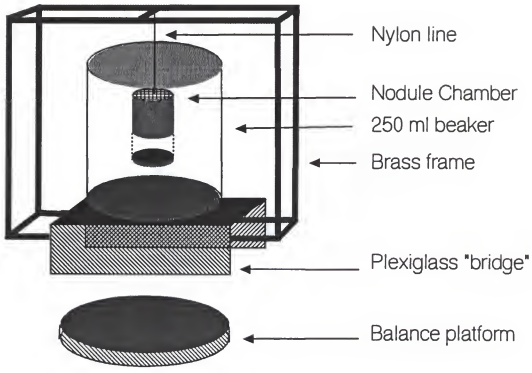

Figure 2-1. Apparatus used to measure bulk nodule density. Brass frame rested on the platform of an analytical balance. A beaker filled with deionized water was suspended over the brass frame and balance platform by a plexiglass bridge. The buoyant force of nodules in the chamber was transmitted to the brass frame and balance platform by the nylon line. For details on calculating density, refer to text.

$$BF = (FW - \Delta IW) \bullet g \qquad (2\text{-}2)$$

where ΔIW is the change in immersed weight of the chamber due to nodules either increasing or decreasing the chamber weight in water, and g is the gravitational constant (980.6 cm s^{-2}). For example, nodules less dense than water would decrease the immersed weight of the chamber and cause ΔIW to be a negative value; BF would therefore be greater than FW.

Buoyant force may also be expressed by the relationship:

$$BF = v \bullet d \bullet g \qquad (2\text{-}3)$$

where v is total nodule volume and d is the density of water at 25°C (0.9977 g cm^{-3}). If equation 2-2 is set equal to equation 2-3, then the gravitational terms cancel, and an equation describing nodule volume can be expressed in terms of known variables:

$$v = (FW - \Delta IW) / d \qquad (2\text{-}4)$$

Bulk nodule density ($\bar{\rho}$) is then calculated as the quotient of FW divided by v.

Intact Plant Experiments

Plants used for the experiments involving intact plants were grown aeroponically in the 38-L aquariums described previously. When plants had developed 3 to 4 fully expanded leaves, the pO_2 entering the aquariums was either kept at 21 kPa or was switched to 10 or 31 kPa for a 24-h period beginning at 1200 h. The pO_2 was decreased to 10 kPa by mixing equal volumetric flows of N_2 and air. The pO_2 was increased to 31 kPa by the addition of O_2 in two steps into a 19-L mixing tank. The flow rate of each air mixture into the aquariums was maintained at 2 L min^{-1}.

After 24 h of the O_2 treatment, five nodules ranging in size from 3 to 4 mm in diameter were removed from the top 10 cm of the root, blotted dry, and frozen in liquid N_2. Sap was expressed from an individual frozen nodule onto a filter disk and placed in a vapor pressure osmometer (Wescor 5500, Logan, UT) to determine bulk nodule osmotic potential ($\bar{\Psi}_\pi$). The remaining nodules on the top 10 cm of the root were removed, blotted dry, and $\bar{\rho}$ determined as described previously.

Nodule Air Space Determination

The fractional air space content of nodules from plants of two treatments was compared: (1) control, aeroponically grown plant with three to four fully expanded leaves was kept under high intensity laboratory lights (2300 μmol m^{-2} s^{-1} PPFD at the top of the plant) on 16 h photoperiod for 2 days; and (2) shaded, receiving less than 100 μmol m^{-2} s^{-1} PPFD for 2 days, but otherwise same as control.

Determination of fractional air space content of nodules was similar to the procedure described for air space determination in root segments of Vicia faba (Evans and Ebert, 1960). Nodules were removed from plants of each treatment and $\bar{\rho}$ determined as described in the previous section. Following $\bar{\rho}$ measurement, nodules were placed in a test tube with deionized water and a 0.05 MPa vacuum was applied to the test tube contents for 10 s. The vacuum was then released and reapplied for an additional 10 s, and this process was repeated for a total of five vacuum infiltrations.

After the final vacuum infiltration, nodules were blotted dry and a second bulk nodule density measurement was made. The increase in density due to vacuum infiltration was assumed to be due to replacement of intercellular air pathways with water. The fractional air space content (ASC) was calculated as follows:

$$ASC = (\Delta m \bullet d) / v \qquad (2\text{-}5)$$

where Δm is the difference between mass of nodules before and after vacuum infiltration, d is the density of water at 25°C (0.9977 g cm^{-3}), and v is the volume of nodules after removal from the plant.

Results and Discussion

Bulk Nodule Density

The density data for detached nodules were highly variable between treatments but showed a trend for $\bar{\rho}$ to increase during the first hour under the 21 kPa O_2 treatment and remain constant thereafter (Figure 2-2). Bulk nodule density of nodules exposed to 10 kPa O_2 was approximately the same from 5 to 240 min following detachment. The ratio of nodule density of the 10 to the 21 kPa O_2 treatments eliminated much of the variation and indicated that at 5 min, $\bar{\rho}$ was the same for the O_2 treatments (Figure 2-3). However, 60 to 240 min after detachment, the ratio of $\bar{\rho}$ for the 10 to 21 kPa O_2 treatment was significantly less than 1.000 and ranged from 0.977 to 0.988. This indicates nodule density was greater for the 21 than the 10 kPa O_2 treatment.

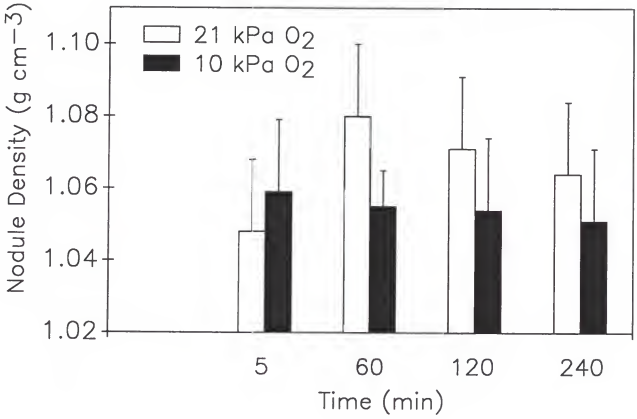

Figure 2-2. Bulk nodule density response to 21 kPa or 10 kPa O_2 treatments in nodules detached for 5, 60, 120, or 240 min. Values reported are the mean and se (n=4).

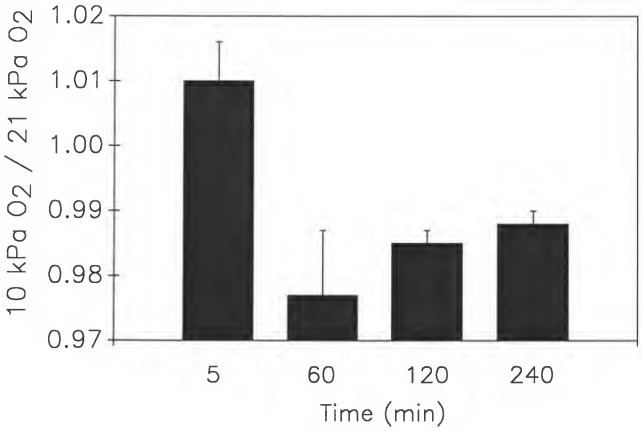

Figure 2-3. Ratio of bulk nodule density of 10 kPa and 21 kPa O_2 treatments in nodules detached for 5, 60, 120, or 240 min. Values reported are the mean and se (n=4).

Sung et al. (1991) found a decrease in P following the detachment and exposure of nodules to ambient pO_2. If there is a similar decrease in P of nodules detached and aerated with ambient pO_2 in deionized water, then this response indicates an inverse relationship between P and $\bar{\rho}$. The displacement of intercellular air with water would increase the diffusion barrier thickness and account for both the decrease in P (equation 1-1) and the increase in $\bar{\rho}$. By a similar argument, P would remain constant following the detachment and aeration of nodules with 10 kPa O_2 since $\bar{\rho}$ remained the same (Figures 2-2, 2-3). No information is available on the effect of sub-ambient pO_2 on P in detached nodules. The 10 kPa O_2 treatment would initially halve the O_2 flux into the nodule (equation 2-1) and decrease the internal pO_2. The internal pO_2 has been proposed to function as a sensor regulating P (Layzell and Hunt, 1990) whereby P increases as internal pO_2 decreases. The lower internal pO_2 at the 10 kPa O_2 treatment might offset the effect of detachment on P.

In contrast to the effect of detachment and O_2 treatment on $\bar{\rho}$, there was no difference in $\bar{\rho}$ after roots and nodules of intact plants were exposed to 10, 21, or 31 kPa O_2 for 24 h (Table 2-1). Similarly, exposure of intact plants to a prolonged shade treatment had no effect on $\bar{\rho}$ when compared to control plants (Table 2-2). The different response of $\bar{\rho}$ to O_2 treatment in detached nodules and intact plants may be due to the magnitude of the P changes associated with each treatment. In intact plants, there is an approximate 2-fold change in P between the 10 and 31 kPa O_2 treatments after 24 h (Weisz and Sinclair, 1987b) and a 2- to 3-fold decrease in P for shaded plants (data not shown). This is in contrast to

Table 2-1. Bulk nodule density and osmotic potential ($\bar{\Psi}_\pi$) response to 10, 21, or 31 kPa O_2 treatment for 24 h in intact plants. Values reported are the mean $\pm$ se.

Experiment	pO_2	Bulk Nodule Density†	$\bar{\Psi}_\pi$‡
	kPa	---g cm^{-3}---	----MPa----
1	10	0.98 $\pm$ 0.02	-1.12 $\pm$ 0.03
	21	0.96 $\pm$ 0.01	-1.17 $\pm$ 0.04
2	10	1.02 $\pm$ 0.01	-1.32 $\pm$ 0.02
	21	1.03 $\pm$ 0.01	-1.35 $\pm$ 0.02
3	10	0.99 $\pm$ 0.01	----------
	21	0.99 $\pm$ 0.01	----------
	31	1.00 $\pm$ 0.01	----------

† n=6
‡ n=30

Table 2-2. Bulk nodule density before and after vacuum infiltration, and fractional nodule air space response in plants shaded for 48 h versus control plants. Values reported are the mean ± se (n=6).

Treatment	Bulk Nodule Density	Bulk Nodule Infiltrated Density	Fractional Nodule Air Space†
	----------g cm^{-3}----------		
Control	0.98 ± 0.01	1.02 ± 0.01	0.046 ± 0.005
Shaded	0.99 ± 0.01	1.02 ± 0.00	0.034 ± 0.006

† Fractional air space was calculated as the difference in mass of nodules before and after vacuum infiltration multiplied by the density of water at 25°C (0.9977 g cm^{-3}) and divided by the volume of the nodules.

an estimated 4-fold decrease in P in the first hour following nodule detachment (calculated from nodule respiration following detachment, from Sung et al., 1991). The larger change in P for detached nodules compared to intact plants would displace a greater volume of intercellular air with water and appreciably alter $\bar{\rho}$.

Although $\bar{\rho}$ of plants under prolonged shade treatment was approximately the same as $\bar{\rho}$ of control plants (Table 2-2), the fractional air space content of nodules for the shade treatment was significantly less than that of the control treatment (Table 2-2). Fractional air space content of nodules may therefore be a more sensitive and accurate measure of intercellular water than $\bar{\rho}$. The decrease in fractional air space content of nodules from shaded plants indicates that there was an increase in the intercellular water content or a decrease in intercellular space. A decrease intercellular air spaces may increase the thickness of a diffusion barrier and decrease P.

Bulk Nodule Osmotic Potential

There was no difference in the response of $\bar{\Psi}_\pi$ following exposure of intact, nodulated roots to 10 or 21 kPa O_2 for 24 h (Table 2-1). It appears that changes in $\bar{\Psi}_\pi$ do not explain P regulation. One possibility accounting for the lack of association between $\bar{\Psi}_\pi$ and P is that the variability between individual nodules was greater than the changes in $\bar{\Psi}_\pi$ required to cause shifts in P. For a given pO_2 treatment, the mean $\bar{\Psi}_\pi$ had a standard error of approximately 0.03 MPa (Table 2-1). Therefore, a change in $\bar{\Psi}_\pi$ of less than 0.03 MPa could not be resolved by psychrometric means. A change in $\bar{\Psi}_\pi$ of 0.03 MPa corresponds to a change in solute concentration of 12 mM (by the Van't Hoff relationship, equation 3-2).

Whether or not a solute concentration change of 12 mM or less would have physiological significance in intercellular air pathway regulation depends upon the localization of the solute within particular cells of the tissue and the water release properties of these cells. In particular, the concentration of solute required to cause a given change in intercellular water content is proportional to the elasticity of the nodule (equation 3-8). Quantification of individual solutes in different regions of the nodule at high versus low permeabilities and determination of nodule elasticity should help resolve the question of solute contribution to P regulation.

The second possibility explaining the lack of association between $\bar{\Psi}_\pi$ and P is that bulk nodule osmotic potential did not change because the absolute amount of solute in the tissue was the same at different permeabilities. Instead of a change in solute concentration there may be a redistribution of solutes from symplast to apoplast. The redistribution of solutes would affect the surrounding cellular osmotic potential but not bulk nodule osmotic potential measured by the freeze-thaw technique. Bulk nodule osmotic potential of a tissue may also be determined by pressure-volume analysis. Pressure-volume analysis avoids the mixing of apoplastic and symplastic fractions inherent in $\bar{\Psi}_\pi$ determination by freeze-thaw methodology. Pressure-volume analysis also allows a determination of water release properties and elasticity of a tissue.

The third possibility explaining the lack of association between $\bar{\Psi}_\pi$ and P is that P changes are, in fact, independent of changes in $\bar{\Psi}_\pi$. Instead of $\bar{\Psi}_\pi$, bulk nodule pressure potential ($\bar{\Psi}_p$) may control the movement of water in intercellular

spaces to regulate P, assuming that the bulk modulus of elasticity changes during P adjustment (equation 1-4). Experimentally, the components of nodule water potential may be affected directly by changing the water status of the entire plant. The responses of $\bar{\Psi}_\pi$ and $\bar{\Psi}_p$ may then be compared to changes in P during water deficit treatments to assess a possible causal relationship.

Results from these experiments indicate that prolonged shading treatments, which cause P to decrease, also result in a decrease in fractional air space content of nodules. This result is consistent with a water-filled diffusion barrier increasing in effective thickness and decreasing P. However, the regulation of P does not appear to be controlled by $\bar{\Psi}_\pi$ as measured by freeze-thaw methodology. The various possibilities for the lack of association between $\bar{\Psi}_\pi$ and P are explored in succeeding chapters.

Experiments in Chapter 3 re-examine the possible influence of nodule water potential components by pressure-volume analysis on P. Pressure-volume analysis allows for the determination of nodule elasticity, bulk nodule pressure potential, and the amount of solute required for given changes in relative volume. Concentration of solutes which may act osmotically in P regulation was also determined from nodules differing in P. If nodules were highly elastic then P changes could be achieved with small solute changes which are below psychrometric resolution.

Experiments in Chapter 4 examine the relationship of total bulk nodule water potential, bulk nodule pressure potential, and bulk nodule osmotic potential on nodule permeability by an approach different from that employed in Chapters

2 and 3. In Chapter 4 plant water status was rapidly altered and the response of P, dinitrogenase activity, nodule respiration, and nodule water potential was followed for a 7 h period. Additionally, a method is described to determine the water release properties of nodules desiccating while attached to the plant. This method complements the pressure-volume determination of detached nodules in Chapter 3.

CHAPTER 3
PRESSURE-VOLUME RELATIONSHIP OF SOYBEAN NODULES AND COMPOSITIONAL ANALYSIS OF NODULES WITH DIFFERING GAS PERMEABILITIES

Introduction

The partial pressure of O_2 (pO_2) surrounding nodules directly affects the permeability (P) of the nodule to gas exchange. A change in the rhizosphere pO_2 from 21 to 10 kPa causes an immediate decrease in dinitrogenase activity and nodule respiration (Weisz and Sinclair, 1987a, 1987b). After 4 to 8 hours at the sub-ambient pO_2, P increases and respiration and dinitrogenase activity return to pretreatment rates. Conversely, 4 to 8 hours following a switch in the pO_2 surrounding nodules from 21 to 31 kPa, P decreases and nodule respiration and dinitrogenase activity resume pretreatment rates (Weisz and Sinclair, 1987a, 1987b).

The mechanism regulating P changes in response to O_2 is not understood but is believed to be associated with varying the thickness of a water-filled diffusion barrier in the nodule cortex. Varying the thickness of a diffusion barrier could be accomplished by cells extracting water from, or releasing water to, intercellular spaces (Figure 1-2). The uptake and release of cellular water will be governed by changes in the components of water potential (equation 1-4). If

shifts in nodule water potential are responsible for changes in P as proposed, this may be examined by exposing nodules to sub- and supra-ambient pO_2's for several hours, in order to cause changes in P (Weisz and Sinclair, 1987a, 1987b), and then comparing the components of water potential for each O_2 treatment.

One method for examining the components of water potential of a tissue ($\bar{\Psi}_{tot}$) is pressure-volume analysis (Koide et al., 1989; Tyree and Jarvis, 1982). Experimentally, $\bar{\Psi}_{tot}$ is determined by either pressure-chamber or psychrometric techniques over a range of tissue hydration levels. Tissue hydration is determined by the cumulative volume of liquid exuded through the xylem as pressure is increased in the pressure-chamber, or by the decrease in relative water content (RWC) as the tissue desiccates. Relative water content is defined as the amount of water in a tissue at any given time relative to the amount of water in a tissue when completely hydrated (Tyree and Jarvis, 1982):

$$RWC = (FW - DW) / (TW - DW) \qquad (3\text{-}1)$$

The terms FW, DW, and TW refer to the fresh, dry, and turgid weights of a tissue, respectively.

A plot of the negative reciprocal of $\bar{\Psi}_{tot}$ versus RWC generally has a large initial decrease in $-\bar{\Psi}_{tot}^{-1}$ for a given change in RWC due to the loss of pressure potential ($\bar{\Psi}_p$) in the tissue (Koide et al., 1989). At the point where $\bar{\Psi}_p = 0$, further decreases in RWC yield a straight line. Theoretically, the intercept of this line with the abscissa is an estimate of the fraction of apoplastic water (R_a). An inverse evaluation of the intercept with the ordinate when RWC=1 is an estimate of the osmotic potential ($\bar{\Psi}_\pi$) at full hydration ($\bar{\Psi}_\pi{}^\circ$) (Koide et al., 1989). The

bulk pressure potential, $\bar{\Psi}_p$, may be calculated for those points lying above the regression line by subtracting an extrapolated value of the osmotic component from the total water potential at a particular RWC.

Variations in nodule water potential associated with P regulation may be due to changes in the accumulation and distribution of solutes in the inner cortex (Figure 1-2). This could regulate P in at least two different ways. First, a release of solutes to the apoplast would cause water to leave cells, fill intercellular spaces with water, and thereby decrease P. An increase in P would result from an uptake or generation of solutes in cells and withdrawal of water from intercellular spaces. Second, solute accumulation would result in cell expansion and the displacement of intercellular pathways, decreasing P. Accordingly, a decrease in cellular solutes would result in a decrease in cell size and an opening in intercellular air spaces. An implicit assumption in both methods of P regulation is that apoplastic water in the variable diffusion barrier is in equilibrium with apoplastic water elsewhere in the nodule. Otherwise, releasing solutes to a diffusion barrier apoplast, which is isolated from the rest of the nodule, would result in an increase in extracellular water that was offset by a decrease in cell size such that the air space volume would remain constant.

The identity of a solute responsible for P regulation has not been examined. Both soluble sugars and ureides have been proposed as functioning as osmoticants regulating P in nodules (Walsh et al., 1989a). Additionally, amino acids are formed from the assimilation of NH_3 produced during dinitrogen

fixation (Miflin and Lea, 1976), and amino acid accumulation may function osmotically in different tissues (Stewart, 1981).

Treatments which lower ureide production lead to a decrease in P. These treatments include exposure to the urate oxidase inhibitor, allopurinol, (Atkins et al., 1988), prolonged exposure to C_2H_2 (Witty et al., 1984), and replacing the N_2:O_2 atmosphere around nodules with Ar:O_2 (Witty et al., 1984). Other treatments, which decrease P but lead to an increase in nodule ureide content, are associated with low rates of phloem supply (Walsh et al., 1989a). A decrease in phloem supply increases ureide content because the water carrying the ureides out of the nodule, in the xylem, is derived primarily from the phloem (Walsh et al., 1989a). The treatments which decrease P but lead to an increase in nodule ureides include: drought stress (Weisz et al., 1985; Sheoran et al., 1981), prolonged darkness (Walsh et al., 1989a) and NO_3^- application (Schuller et al., 1988; Streeter, 1985; Vessey et al., 1988).

Walsh et al. (1989a) have suggested that under conditions of decreased phloem supply, and therefore low ureide export rates, the symplastic sucrose concentration in the nodule cortex will decrease while the apoplastic ureide concentration will increase. This scenario may lead to water filling intercellular air spaces and may account for the decrease in P under conditions of drought (Weisz et al., 1985), NO_3^- application (Schuller et al., 1988; Vessey et al., 1988), and prolonged darkness (Walsh et al., 1989a). Preliminary reports indicate that the apoplastic ureide concentration increases following nodule detachment (Streeter, 1992), and this treatment is associated with a decrease in P (Sung et al.,

1991). However, the proposal of Walsh et al. (1989a) does not explain the parallel decreases in nodule ureide concentration (Walsh et al., 1989a) and P (Witty et al., 1984) associated with exposure of nodules to a N_2-free atmosphere.

The objectives of the experiments described in this chapter were to: (1) establish a pressure-volume curve for nodules differing in P, and from the pressure-volume data separate the components of $\bar{\Psi}_{tot}$; (2) determine if the water release properties of nodules are favorable for the regulation of P by the release and uptake of water; and (3) quantify three classes of compounds, ureides, sugars, and free amino acids, from nodules differing in P and which might act osmotically in the regulation of P.

Analysis of Nodule Water Relations

The water potential of a cell (Ψ_{tot}, MPa) was described in Chapter 1 as the sum of the pressure (Ψ_p) and osmotic (Ψ_π) potentials (equation 1-2):

$$\Psi_{tot} = \Psi_p + \Psi_\pi$$

An isolated cell, without turgor, behaves as an osmometer, and Ψ_π may be expressed by the Van't Hoff relationship (Nobel, 1991):

$$\Psi_\pi = -(sRT)/v \qquad (3\text{-}2)$$

where s is the amount of solute (osmoles), v is the volume of water in the cell (L), R is the gas constant, and T is the temperature ($^\circ$K). Assuming that the density of water is 1 kg/L, concentration in equation 3-2 is given in osmoles/kg. However, molarity may be more convenient to work with than molality, and for dilute solutions (less than 0.2 M), osmolality and osmolarity (osmoles/L) are

approximately the same (Nobel, 1991). Given these assumptions and approximations, equation 3-2 may be substituted into the equation describing Ψ_{tot}:

$$\Psi_{tot} = \Psi_p - (sRT)/v \qquad (3\text{-}3)$$

If equation 3-3 is solved for Ψ_p and differentiated with respect to s, then Ψ_{tot} drops out of the equation, and an expression is given relating changes in pressure to changes in solutes:

$$d\Psi_p = RT \bullet ds/v \qquad (3\text{-}4)$$

Changes in Ψ_p depend upon the product of the elastic modulus (ϵ, MPa) and changes in relative volume (dv/v; equation 1-5). The elastic modulus is a coefficient describing the rigidity of a cell, and cells with a high elastic modulus are inelastic. The term ($dv/v \bullet \epsilon$) may replace $d\Psi_p$ in equation 3-4:

$$dv/v \bullet \epsilon = RT \bullet ds/v \qquad (3\text{-}5)$$

Equation 3-5 may be solved for ds/v to give:

$$ds/v = \epsilon/RT \bullet dv/v \qquad (3\text{-}6)$$

Equation 3-6 indicates that for a given change in relative volume, the change in concentration is proportional to the elastic modulus.

Water relations in a tissue, such as a nodule, are similar to the water relations of an individual cell. By analogy to the water relations of an individual cell, the bulk modulus of elasticity ($\bar{\epsilon}$, MPa) may be described by the following equation:

$$\bar{\epsilon} = \Delta\bar{\Psi}_p \,/\, (\Delta RWC - R_a) \qquad (3\text{-}7)$$

where $\Delta\bar{\Psi}_p$ is the change in bulk nodule pressure potential (MPa), ΔRWC is the change in relative water content, and R_a is the fraction of apoplastic water. The

denominator in equation 3-7 represents the symplastic relative water content (RWC_{sm}) and is analogous to relative cell volume ($\Delta v/v$; equation 1-5). Equation 3-7 indicates that inelastic tissues with a high value of $\bar{\epsilon}$ undergo a relatively large drop in $\bar{\Psi}_p$ for a given change in RWC_{sm} compared with elastic tissues with a low value of $\bar{\epsilon}$.

A finite form of equation 3-6 may also be expressed on the tissue level basis:

$$\Delta s/v = \bar{\epsilon}/RT \bullet \Delta RWC \qquad (3\text{-}8)$$

By knowing $\bar{\epsilon}$, the concentration of solute required to cause a shift in the symplastic water content can be determined.

Water release from a tissue also depends upon the intrinsic capacitance (C_j, MPa^{-1}) of that tissue (Tyree and Jarvis, 1982):

$$C_j = \Delta RWC \,/\, \Delta\bar{\Psi}_{tot} \qquad (3\text{-}9)$$

The intrinsic capacity is associated with the ability of a tissue to release water with a given change in $\bar{\Psi}_{tot}$. If C_j is known, then the amount of water released for a given change in $\bar{\Psi}_{tot}$ can be determined from equation 3-9. The intrinsic capacitance may also be expressed in terms of $\bar{\epsilon}$ and $\bar{\Psi}_\pi$ (Tyree and Jarvis, 1982):

$$C_j = RWC_{sm} \,/\, (\bar{\epsilon} - \bar{\Psi}_\pi) \qquad (3\text{-}10)$$

Materials and Methods

Plant Growth Conditions

Soybean seedlings (cv. Biloxi) were removed from an artificial soil medium (Terr-lite, WR Grace Co., Cambridge, MA) 3 to 4 days after sowing, and the root system was passed through one of three holes in a #7 rubber stopper. The plant-

stopper combination was placed in the lid of a 1.5-L aeroponic chamber filled with half-strength, N-free Hoagland's solution (Hoagland and Arnon, 1938). Plants were inoculated with a commercial preparation of Bradyrhizobium japonicum (Nitragin Co., Milwaukee, WI), and grown in the greenhouse under natural illumination with the photoperiod extended to 16 h with incandescent lamps.

Plants used for nodule compositional analysis were grown similarly to those in the pressure-volume study except the roots of six to eight plants were grown in common 38-L aquariums. The aquariums were filled with half-strength, N-free Hoagland's solution (Hoagland and Arnon, 1938), and each aquarium was continuously aerated with ambient air (79:21, N_2:O_2) at a flow rate of 2 L min^{-1}. Aquariums were placed inside a thermally insulated chamber to modulate root and nodule temperature.

Pressure-Volume Determinations

Three days prior to measurement of nodule water relations, a plant with three to four fully expanded leaves was removed from the aeroponic chamber and transferred to a 500-ml Erlenmeyer flask containing half-strength, N-free Hoagland's solution (Hoagland and Arnon, 1938). Nutrient solution was aerated through one of three holes in the stopper supporting the plant. The evening before the measurement, the plant was brought to the laboratory at 1800 h and sealed into a glove box. The nutrient solution in the Erlenmeyer flask was aerated at a flow rate of 0.2 L min^{-1} for a 12-h period with either a 21 kPa O_2

supply (ambient air) or a 10 kPa O_2 supply. Exposure of nodules to 10 kPa O_2 for 12 h increased P approximately 150% (Weisz and Sinclair, 1987b).

The O_2 treatments in the pressure-volume experiments were designed to alter nodule permeability. Conducting the experiments within the glove box ensured that the pO_2 during the pressure-volume determination was the same as the pO_2 during the previous night.

At 0600 h the following morning, pressure-volume determination on nodules was begun. The entire pressure-volume procedure was conducted inside the glove box at the specified pO_2. Five nodules, 2.5 to 3.0 mm in diameter, were removed from the upper 10 cm of the root, blotted dry, weighed and sealed into a vapor pressure osmometer (Wescor 5500). After vapor pressure equilibrium had been established (45 to 90 min), $\bar{\Psi}_{tot}$ was measured. The nodules were placed on the balance and allowed to desiccate to an estimated RWC (equation 3-1), and then the nodules were placed back in the osmometer for $\bar{\Psi}_{tot}$ determination. This process was repeated six to seven times during the day. After the final $\bar{\Psi}_{tot}$ measurement, nodules were placed in a dryer at 80°C for 72 h to determine dry weight. Nodules from four plants of both O_2 treatments were used in the pressure-volume analysis.

Compositional Analysis Experiments

Experimental treatments and plant harvesting

Six separate experiments were conducted on plants with four to five fully expanded leaves to determine soluble sugars, free amino acids, and ureides from nodules differing in permeability properties. Exposure of nodules to 10 kPa or 31

kPa pO_2 caused a respective increase or decrease in P (Weisz and Sinclair, 1987b). In experiments 1 through 4, the aquariums containing roots of six plants were randomly assigned as either 21 kPa or 10 kPa O_2 treatments. In addition, each plant within an O_2 treatment was randomly designated to be harvested at 1, 2, or 24 h after treatment initiation. In experiment 5, rhizospheric O_2 treatments of 10 kPa and 31 kPa were implemented and harvests were made at 1 and 24 h after treatment initiation on 4 plants per O_2 treatment per harvest. In experiment 6, rhizospheric O_2 treatments of 10, 21, and 31 kPa were examined at 24 h after treatments began. Ureide content of nodules in experiment 6 was approximately one-third that of the other experiments, and lower than values reported in the literature. For these reasons, ureides in experiment 6 were not included in the results.

In each of the experiments, O_2 treatments began at 1200 h. For the 10 kPa O_2 treatment, equal volumetric flows of N_2 and ambient air were mixed. For the 31 kPa O_2 treatment, the O_2 concentration in ambient air was increased by adding O_2 to increase the pO_2 to 26 kPa . After 30 minutes at 26 kPa the pO_2 was increased to 31 kPa. A 19-L mixing tank was placed between the source of the gases and the aquariums for the supra-ambient treatment. At a flow rate of 2 L min^{-1}, the increase in O_2 concentration did not exceed 0.2 kPa min^{-1}.

At the appropriate harvest interval, a plant was removed from the chamber, nodules were removed from the upper 10 cm of the root, blotted dry, weighed, and frozen in liquid N_2. In experiments 1 and 2, nodules greater than 2 mm in diameter were harvested; in experiments 3 and 4, nodules were separated

into large (>3 mm diameter) and small (<3 mm diameter) size classes. The volume of cortical and infected nodule tissues in each size class was estimated by photographing the frozen nodules along side a scaled ruler, measuring the longest nodule dimension in the projected photograph. From these measurements, total nodule volume and infected region volume were calculated using regression equations (Weisz et al., 1985; Weisz and Sinclair, 1988a), and cortical volume was calculated by difference.

In experiments 5 and 6, nodules greater than 2 mm were harvested and freeze-dried. Freeze-dried nodules were viewed on the platform of a dissecting scope at 10x. By holding the nodule with forceps, the cortical tissue was carefully separated from the infected region with a scalpel. In general, the cortical and infected tissues separated easily at their junction. The separated infected tissue appeared covered with a whitish layer that was distinct from the pinkish color of the remaining infected tissue. The whitish layer may have been composed of a few cells layers of the inner cortex that adhered to the infected region during separation.

Nodule extraction

Between 100 and 300 mg of frozen nodules in experiments 1 through 4 were extracted by grinding frozen nodules in 5 ml of hot 80% ethanol in a tissuemizer (Tekar, Cincinnati, OH), centrifuging the homogenate at 4500g for 15 minutes, and collecting the supernatant. The pellet was resuspended two additional times in 5 ml of water, heated for 10 minutes at 80°C, centrifuged, and

the supernatants pooled. The extract was filtered through Whatman #5 filter paper and stored at 4°C.

A 35-mg sample of freeze-dried nodule tissues from the cortical or infected nodule regions was extracted by adding 5 ml of water to the sample and boiling for 30 minutes, grinding in a tissuemizer for 30 seconds, and placing the sample in a boiling water bath for 2 hours. The sample was then filtered, brought to volume, and a 20-ml subsample was stored at -20°C.

Chemical analysis

Soluble sugars and ureides were determined from nodule extracts in all experiments, and free amino acids were determined from nodule extracts in experiments 1 through 4. Soluble sugars were measured using the phenol-H_2SO_4 procedure of Nalewaja and Smith (1963) against glucose standards. Ureides were measured as the phenylhydrazine derivative against allantoin standards following the procedure of Young and Conway (1942). Free amino acids were determined by the ninhydrin-KCN procedure of Yem and Cocking (1955) against glycine standards. Proline reacts differently with ninhydrin than the other amino acids and has a different absorption peak. Proline levels were not quantified and are not included in the reported free amino acids.

Results

Pressure-Volume Determinations

The negative reciprocal of total bulk water potential ($-\bar{\Psi}_{tot}^{-1}$) was plotted against RWC, and visual inspection of the curve indicated that points with a RWC

less than 0.85 were approximately linear (Figure 3-1). Linear regression equations by O_2 treatments were calculated for points with a RWC less than 0.85, and these equations were not statistically different (Table 3-1). A regression equation combining O_2 treatments for points with a RWC less than 0.85 had a range or residuals squared from 0.000 to 0.051. It was assumed that if points with a RWC>0.85 fell on the same regression line, then the residuals squared would fall within the same range as points with RWC<0.85. This selection procedure was used to determine which points to include in the regression equation presented in Figure 3-1.

The hypothesis presented in this study was that P adjustment may be due to changes in $\bar{\Psi}_\pi$ or $\bar{\Psi}_p$. However, treatment of nodules with 10 or 21 kPa O_2 had no effect on $\bar{\Psi}_\pi$ or $\bar{\Psi}_p$ (Table 3-1, Figure 3-1, Figure 3-2). The reason for the lack of differences in $\bar{\Psi}_\pi$ and $\bar{\Psi}_p$ between O_2 treatments may be because the effects of nodule detachment and desiccation in this experiment dominated over O_2 treatment effects in the pressure-volume measurements. Within 5 minutes of detachment, specific dinitrogenase activity of nodules is 50% of intact plants (Sung et al., 1991). Dinitrogenase activity may be restored by increasing the pO_2 surrounding nodules to 50 kPa, indicating that detachment decreases P (Ralston and Imsande, 1982). Desiccation of detached nodules also causes a further decrease in P compared to well-hydrated, detached nodules (Pankhurst and Sprent, 1976). Combining the O_2 treatments, the regression equation in Figure 3-1 predicted $\bar{\Psi}_\pi{}^\circ$=-0.48 MPa and R_a=0.28.

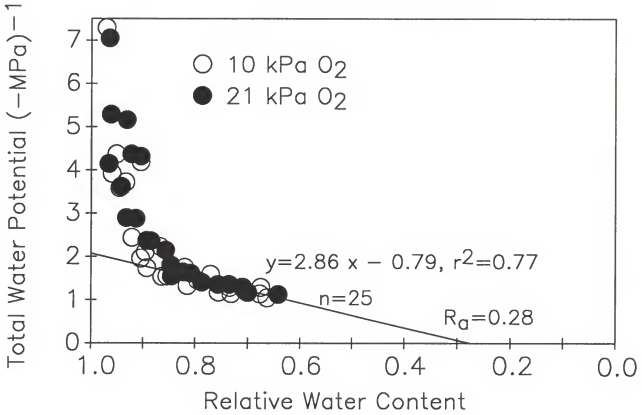

Figure 3-1 Inverse plot of total water potential versus relative water content (RWC) for detached soybean nodules measured under 10 or 21 kPa O_2. Regression equation is over O_2 treatments. The x-intercept represents the apoplastic water fraction (R_a). Inverse evaluation of the regression equation when RWC=1 indicates that osmotic potential at full hydration ($\bar{\Psi}_\pi^\circ$) was -0.48 MPa.

Table 3-1. Regression equations for pressure-volume analysis by O_2 treatment of: the negative inverse of total water potential ($-\Psi_{tot}^{-1}$) versus relative water content (RWC); bulk nodule pressure potential ($\bar{\Psi}_p$) vs RWC; and $\bar{\Psi}_p$ vs $-\bar{\Psi}_{tot}$.

dependent variable	independent variable	O_2 treatment	slope ± se	y intercept ± se	n	r^2
		---kPa---				
$-\bar{\Psi}_{tot}^{-1}$	RWC†	10	2.76 ± 0.90	-0.73 ± 0.16	10	0.54
$-\bar{\Psi}_{tot}^{-1}$	RWC†	21	3.02 ± 0.38	-0.87 ± 0.08	11	0.88
$-\bar{\Psi}_{tot}^{-1}$	RWC‡	pooled	2.86 ± 0.33	-0.79 ± 0.12	25	0.77
$\bar{\Psi}_p$	RWC	10	1.83 ± 0.76	-1.47 ± 0.07	9	0.46
$\bar{\Psi}_p$	RWC	21	1.68 ± 0.54	-1.31 ± 0.06	12	0.49
$\bar{\Psi}_p$	RWC	pooled	1.72 ± 0.43	-1.35 ± 0.06	21	0.46
$\bar{\Psi}_p$	$-\bar{\Psi}_{tot}$	10	-0.80 ± 0.06	0.47 ± 0.02	9	0.96
$\bar{\Psi}_p$	$-\bar{\Psi}_{tot}$	21	-0.77 ± 0.05	0.47 ± 0.02	12	0.96
$\bar{\Psi}_p$	$-\bar{\Psi}_{tot}$	pooled	-0.78 ± 0.04	0.47 ± 0.02	21	0.96

† regression equations are for points with RWC < 0.85.

‡ regression equation is for points with RWC < 0.85 and for those points with RWC > 0.85 which fall on the same line (as described in Results).

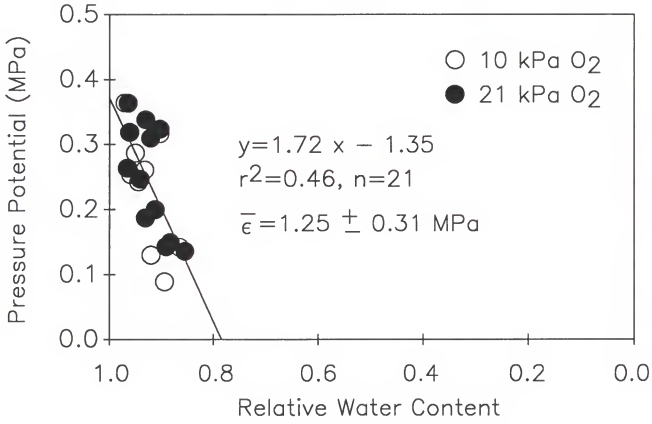

Figure 3-2. Pressure potential versus relative water content for detached soybean nodules measured under 10 or 21 kPa O_2. Regression equation is over O_2 treatments.

Pressure-volume analysis did reveal important mechanical aspects regarding water release properties of nodules. Changes in $\bar{\Psi}_p$ from 0.09 to 0.36 MPa with RWC were approximately linear ($r^2=0.46$) (Figure 3-2), and O_2 treatment did not significantly affect the regression equation (Table 3-1). Although the correlation coefficient was relatively low in these experiments, preliminary pressure-volume analysis of nodules also determined a linear relationship between $\bar{\Psi}_p$ and RWC with a $r^2=0.62$, $n=12$ (data not shown). The linear nature of $\bar{\Psi}_p$ over the range of RWC from 1 to 0.85 indicates that the bulk modulus of elasticity, $\bar{\epsilon}$, was constant and may be calculated by multiplying the slope and the symplastic water content ($1-R_a$, equation 3-7). The curvilinear relationship of $\bar{\epsilon}$ with $\bar{\Psi}_p$ that is often observed as $\bar{\Psi}_p$ approaches zero (e.g., Tyree and Jarvis, 1982) was not apparent in these experiments, although curvilinearity may have occurred at pressure potentials less than 0.09 MPa. For detached nodules, $\bar{\epsilon}$ had a value of 1.25 ± 0.31 MPa (Figure 3-2).

The decrease in $\bar{\Psi}_p$ as $\bar{\Psi}_{tot}$ decreased was also a linear function (Figure 3-3), and the relationship was independent of O_2 treatment (Table 3-1). Extrapolation of the regression line to the abscissa and the ordinate gives 2estimates of the turgor loss point and $\bar{\Psi}_p$ at full hydration ($\bar{\Psi}_p^{\circ}$), respectively. The relationship between $\bar{\Psi}_p$ and $\bar{\Psi}_{tot}$ in Figure 3-3 indicates that turgor was lost at $\bar{\Psi}_{tot}=-0.60$ MPa. Evaluating the inverse of the turgor loss point ($0.60^{-1}=1.65$) with the transformed regression equation developed in Figure 3-1 indicates that turgor was lost at a RWC of 0.85. Extension of the regression line in Figure 3-3

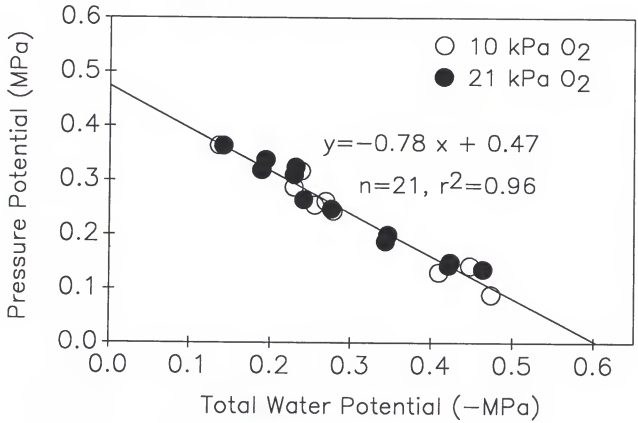

Figure 3-3. Pressure potential versus total water potential for detached soybean nodules measured under 10 or 21 kPa O_2.

to the ordinate indicates that $\bar{\Psi}_p^{\circ}$=0.47 MPa, and this value agrees well with the predicted $\bar{\Psi}_{\pi}^{\circ}$ of -0.48 MPa from Figure 3-1.

A fourth property from the pressure-volume data that is more difficult to quantify is the changes in solute concentration that are required for a given change in relative volume (equation 3-8). For guard cells this relationship is relatively straight forward (equation 3-6). Volume changes in the guard cell system are conceptualized as an opening of K^+ channels, leading to an efflux of K^+ to the apoplast, which causes water to leave the cell and cell volume and turgor to decrease. These series of events ultimately result in stomatal closing (Serrano and Zeiger, 1989). To extend this analogy to the tissue level and apply it to the pressure-volume data violates two assumptions of pressure-volume theory: first, that apoplastic water content is constant, and second, that apoplastic solute concentration is negligible (Koide et al., 1989; Tyree and Jarvis, 1982). However if it is assumed that the value of $\bar{\varepsilon}$ is independent of the pressure-volume determination, $\bar{\varepsilon}$ may then be used to estimate the change in solute concentration required for a given change in symplastic volume.

In the case of the inner cortex of soybean, there is a 40 to 50 μm region comprising approximately three cell layers and which lacks intercellular spaces (Dakora and Atkins, 1991). This region presumably serves as a fixed diffusion barrier. Estimates of extracellular voids in the inner cortex range from 1.7% on an area basis (Parsons and Day, 1990) to 4.1% on a volume basis (Dakora and Atkins, 1991), and varying extracellular water content in these layers may function as the variable component of the diffusion barrier. To completely fill or empty an

extracellular void of 1.7 to 4.1% in a tissue with $\bar{\varepsilon}$ of 1.25 MPa at 25°C requires a change in solute concentration from the symplast to apoplast of 8.7 to 20.4 mM (equation 3-8). A change in solute concentration of this magnitude at 25°C corresponds to a change in osmotic potential of 0.02 to 0.05 MPa (equation 3-2).

The amount of water lost from a tissue for a given change in $\bar{\Psi}_{tot}$ depends upon the intrinsic capacitance of that tissue (Tyree and Jarvis, 1982). The intrinsic capacitance (C_j, MPa^{-1}) may be defined as the change in RWC for a given change in $\bar{\Psi}_{tot}$ (equation 3-9). The decrease in $\bar{\Psi}_{tot}$ of nodules with decreasing RWC was linear from full hydration (RWC=1) to the turgor loss point (RWC=0.85) with a slope of 3.46 MPa (Figure 3-4). The inverse of this slope represents C_j (Koide et al., 1989), and has a value of 0.29 MPa^{-1}. Alternatively, if C_j is calculated from equation 3-10 assuming a RWC=1, R_a=0.28, $\bar{\varepsilon}$=1.25 MPa, and $\bar{\Psi}_\pi$=-0.48 MPa, then C_j=0.42 MPa^{-1}. These values of C_j for nodules are high and similar to C_j from leaves of desert perennials (Hunt and Nobel, 1987) and stem of palm (Holbrook and Sinclair, 1992). There are few reports of C_j for non-xerophytic plants, but using equation 3-10 C_j was estimated at 0.03 to 0.07 MPa^{-1} for oak leaves (Kubiske and Abrams, 1991), 0.01 MPa^{-1} for hydrated cabbage leaves (Weisz et al., 1989), and 0.03 MPa^{-1} for soybean leaves (Zur et al., 1981). Tissues with a high value of C_j often function to buffer tissue water loss when transpirational demand exceeds supply from the soil.

The high intrinsic capacity of the nodule may allow water to move between the cortical symplast and apoplast with only minor effects on $\bar{\Psi}_{tot}$. Given a C_j in nodules from 0.29 to 0.42 MPa^{-1}, to completely fill or empty the estimated 1.7 to

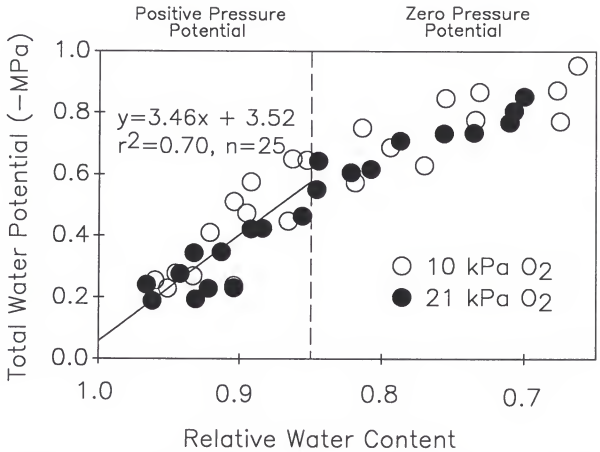

Figure 3-4. Total water potential versus relative water content (RWC) for detached soybean nodules measured under 10 or 21 kPa O_2. Regression equation is over O_2 treatments and for points with a positive pressure potential (i.e., RWC>0.85).

4.1% intercellular air space in the inner cortex (Dakora and Atkins, 1991; Parsons and Day, 1990) would require only a 0.04 to 0.14 MPa change in $\bar{\Psi}_{tot}$ (equation 3-9).

Compositional Analysis

Nodule soluble sugars ranged from 13.9 to 18.6 mg gfw^{-1} (Table 3-2), and nodule ureides ranged from 1.6 to 4.4 μmol gfw^{-1} (Table 3-2). The sugar and ureide content was approximately twice as great for the infected region as the cortical region (Tables 3-3, 3-4). Reported values of nodule soluble sugars and ureides are highly variable and dependent upon photoperiod (Walsh et al., 1987; Swaraj et al., 1988), atmospheric CO_2 (Finn and Brun, 1982; Williams et al., 1982), phloem supply (Walsh et al., 1987, 1989a), and plant water status (Fellows et al., 1987; Khama-Choppra et al., 1984; Sheoran et al., 1981). If a 4:1 fresh weight to dry weight ratio is assumed for control plants in the studies mentioned above, the reported range of soluble sugars was from approximately 3 (Williams et al, 1982) to 28 mg gfw^{-1} (Khama-Chopra et al., 1984) and the range for nodule ureides was from 1.4 (Atkins et al., 1990) to 6 μmol gfw^{-1} (Walsh et al., 1989a). Nodule soluble sugars and ureides from experiments in this report (Tables 3-2, 3-3, and 3-4) fell within these ranges.

Solute accumulation in nodules differing in P was not apparent in the soluble sugar or free amino acid fractions (Table 3-2, Table 3-3). In experiments 1 through 4, nodules exposed to different O_2 treatments for 1, 2, or 24 hours did not differ significantly in soluble sugars or free amino acids (Table 3-2). Nodules in experiments 1 and 2 tended to have higher soluble sugar content than nodules

Table 3-2. Soluble sugars, free amino acids, ureides, and per cent cortical tissue in nodules after exposure to 21 kPa or 10 kPa O_2 for 1, 2, or 24 hours.

experiment (nodule size)	time after start of O_2 treatment	soluble sugars		free amino acids		ureides		fraction of sample composed of cortex	
		21 kPa	10 kPa	21 kPa	10 kPa	21 kPa	10 kPa	21 kPa	10 kPa
	---h---	----------mg gfw^{-1}----------				--μmol gfw^{-1}--			
1 & 2	1	18.6	18.1	2.0	2.1	3.6	3.0	-	-
	2	18.3	18.5	2.0	2.3	3.7	3.3	-	-
	24	17.4	18.5	2.0	2.1	3.3	4.4	-	-
LSD (0.05)		NS		NS		0.7			
3 & 4 (large)	1	15.4	16.4	1.9	1.9	2.0	1.7	0.41	0.43
	2	15.6	16.4	1.9	1.9	3.0	2.7	0.39	0.38
	24	14.7	14.8	2.2	2.2	2.5	3.2	0.39	0.37
LSD (0.05)		NS		NS		0.8		NS	
3 & 4 (small)	1	14.4	15.7	1.8	1.8	1.9	1.4	0.63	0.64
	2	16.3	15.9	1.8	1.8	2.7	2.4	0.63	0.59
	24	14.3	13.9	1.9	2.0	1.6	2.2	0.66	0.55
LSD (0.05)		NS		NS		0.7		NS	

Table 3-3. Soluble sugars of the cortical and infected regions of nodules following exposure to 10 kPa, 21 kPa or 31 kPa O_2 for 1, 2, or 24 hours. Values reported are the mean ± se (n=4 for experiment 5 and n=8 for experiment 6).

experiment	time after start of O_2 treatment	cortical region			infected region		
		10 kPa O_2	21 kPa O_2	31 kPa O_2	10 kPa O_2	21 kPa O_2	31 kPa O_2
	----h----	----------mg gdw^{-1}----------					
5	1	39.4 ± 2.9	-	40.7 ± 1.0	88.0 ± 3.0	-	91.1 ± 3.9
	24	38.4 ± 0.8	-	38.3 ± 1.8	81.3 ± 2.8	-	90.7 ± 2.8
6	24	44.7 ± 2.0	38.0 ± 1.0	39.1 ± 1.6	87.1 ± 1.7	80.8 ± 1.7	85.4 ± 3.7

Table 3-4. Ureides of the cortical and infected regions of nodules following exposure to 10 kPa or 31 kPa O_2 for 1 or 24 hours of treatment. Values reported are the mean ± se (n=4).

experiment	time after start of O_2 treatment	cortical region		infected region	
		10 kPa O_2	31 kPa O_2	10 kPa O_2	31 kPa O_2
	----h----	----------μmol gdw^{-1}----------			
5	1	6.3 ± 1.0	6.2 ± 0.3	12.9 ± 1.0	14.2 ± 0.5
	24	7.4 ± 0.7	7.7 ± 0.5	18.8 ± 1.4	15.7 ± 0.7

from experiments 3 and 4, and the large size class in experiments 3 and 4 tended to have higher soluble sugar content than the small size class. In experiments 5 and 6, soluble sugar content in the infected region was approximately twice as great as soluble sugar content in the cortex (Table 3-3). There was little difference in treatment effects except for an unexplained decrease in soluble sugars in the low O_2 treatment of infected tissue at 24 hours. These results differ from a recent model of P regulation which predicts an increase in sucrose concentration under sub-ambient O_2 levels due to starch hydrolysis in the inner cortex (Hunt et al., 1990).

Nodule ureide content of the ambient O_2 treatment in experiments 1 and 2 was relatively stable throughout the course of the experiment (Table 3-2). The variability in ureide content of the ambient O_2 treatment in experiments 3 and 4 may have been due to measurement of C_2H_2 reduction activity (data not shown) at -0.5, 3, and 23.5 hours after the start of the experiments. Although the flow of C_2H_2 through the chambers was limited to 5 minutes, subsequent experiments indicated that small amounts of C_2H_2 were being released from the nutrient solution even after 60 minutes. The presence of C_2H_2 just prior to the 1 and 24 h harvests may have led to a decrease in ureide content by competing with the reduction of N_2 and decreasing the flow of metabolites through the ureide biosynthetic pathway. Comparisons of ureide content in experiments 3 and 4 should therefore be restricted to O_2 treatments at a given harvest.

Nodule ureide content in experiments 1 through 4 was responsive to a decrease in pO_2. One hour following a change in the pO_2 from 21 kPa to 10 kPa,

nodule ureide content decreased (Table 3-2). The trend for decreasing ureide content at 10 kPa O_2 compared to 21 kPa O_2 after one hour of treatment may reflect the decrease in dinitrogenase activity that occurred after a decrease in pO_2 and before P begins to increase (Weisz and Sinclair, 1987a, 1987b). Additionally, the enzyme responsible for the final step in ureide synthesis, urate oxidase (E.C. 1.7.3.3), requires molecular O_2 and has a poor affinity for O_2 (Woo et al., 1981). Decreasing rhizosphere pO_2 would place constraints on urate oxidase activity and decrease ureide synthesis.

Ureide content for the 10 kPa O_2 treatment increased slightly from the 1 h to the 2 h measurement periods (Table 3-2). At 2 h after the start of treatments, ureide content for the 10 kPa treatment was similar to the ureide content of the ambient O_2 treatment (Table 3-2). The increase in ureide content of the 10 kPa O_2 treatment from 1 to 2 hours of treatment may reflect an increase in P and an associated increase in dinitrogenase activity and ureide biosynthesis.

After 24 h of exposure to 10 kPa O_2, nodule respiration and dinitrogenase was similar to the ambient treatment due to an increase in P (Weisz and Sinclair, 1987a, 1987b). Despite similar rates of dinitrogenase activity after 24 h of treatment, ureide content of the 10 kPa O_2 treatment relative to the 21 kPa treatment ranged from 128 to 138% (Table 3-2).

Ureide content of the infected region in experiment 5 was also approximately twice as great as the cortical region (Table 3-4). While no ambient O_2 treatment was included in experiment 5, ureide content in the infected region followed a pattern similar to that in experiments 1 through 4. One hour after

treatment initiation there was a decrease in ureides of the 10 kPa O_2 treatment compared to the 31 kPa O_2 treatment, and at 24 hours the sub-ambient O_2 treatment had a greater ureide content than the supra-ambient treatment.

Discussion

Pressure-volume analysis indicated that water release properties of the bulk nodule were favorable for the osmotic regulation of P. The elastic nature of nodules ($\bar{\epsilon}$ = 1.25 MPa) could allow small changes in solute distribution to move water from the symplast to the apoplast (equation 3-8). The $\bar{\epsilon}$ of 1.25 MPa for soybean nodules was in comparison to reported values of $\bar{\epsilon}$ ranging from 8 MPa for leaves from Quercus species (Kubiske and Abrams, 1991) to 150 MPa for wheat leaves (Campbell et al., 1979). The low value of $\bar{\epsilon}$ also resulted in a high intrinsic capacitance, C_j, for the bulk nodule (equation 3-10). The high value of C_j allowed a relatively large change in volume for a small change in $\bar{\Psi}_{tot}$. It should be recognized, however, that the bulk nodule is not a homogeneous tissue with respect to cell types, and water release properties of cells in the diffusion barrier may differ from those of the bulk nodule.

The physiological significance of the accumulation of ureides under low O_2 conditions is uncertain. If it is assumed that the increase in ureide concentration is confined to the presumptive diffusion barrier comprising a 60 μm thick concentric shell immediately outside of the infected region (Weisz and Sinclair, 1988a), then the change in ureide concentration observed in these experiments is adequate in emptying water from intercellular air spaces occupying a relative volume of approximately 2% (data not shown). Results from experiment 5

indicated that the increase in ureide content at 24 h was limited to the infected region, and that the cortical ureide content was approximately constant across O_2 concentrations (Table 3-4). However, dissection of freeze-dried nodules regions may have resulted in removal of one to two cells layers of the inner cortex with the infected zone and these cell layers of the inner cortex may have contained a high level of accumulated ureides.

After 24 h exposure to 10 kPa O_2, dinitrogenase activity, and nodule respiration were similar to the 21 kPa O_2 treatment, and P was increased 40 to 50% above the control (Weisz and Sinclair, 1987a, 1987b). Nodule ureide content of the 10 kPa O_2 treatment after 24 h was approximately 130% of the 21 kPa O_2 treatment (Table 3-2) while soluble sugar content remained unchanged. These results are in disagreement with the proposal of Walsh et al. (1989a) that an increase in nodule ureides are associated with restricted phloem supply and low P conditions.

In order for ureides to have acted osmotically and increased P under conditions of the 10 kPa O_2 treatment, they must either be: (a) symplastic to pull water out of intercellular air spaces, or (b) apoplastic such that a decrease in cell size "opens up" intercellular air spaces. Since the vascular bundles in nodules are enclosed within an endodermis, in order for ureides to enter the xylem for export, they must be translocated across the endodermis symplastically (Walsh et al., 1989b). This would tend to favor (a), above, for the regulation of P.

The mechanism leading to an increase in ureide concentration under low O_2 conditions remains in question but may be due to an oxygen limitation in the

vascular bundles. The vascular bundles are enclosed within an endodermis and three layers of pericycle cells (Walsh et al., 1989b). The tight arrangement of cells surrounding the vascular bundles may impose a barrier to O_2 diffusion similar to vascular bundles in the seed coat of soybean (Sinclair, 1988). Loading of the xylem elements with ureides is against a concentration gradient and requires energy (Walsh et al., 1989b). The pericycle cells in the vascular bundles are thought to function as transfer cells, and low O_2 conditions may limit respiration and the energy available to load the xylem. Conversely, supra-ambient O_2 concentrations may increase respiration within the vascular bundle and the energy available to load the xylem. This would decrease the concentration of cellular solutes, allow water to fill intercellular air spaces, and thereby decrease P.

CHAPTER 4
GAS EXCHANGE, WATER POTENTIAL, WATER RELEASE PROPERTIES, AND SOLUTE ANALYSIS OF SOYBEAN NODULES IN RESPONSE TO WATER DEFICITS

Introduction

The gaseous diffusion barrier in soybean nodules responds to environmental signals. Under conditions of supra-ambient O_2 (Weisz and Sinclair, 1987b), shading or detopping (Layzell and Hunt, 1990), and drought (Durand et al., 1987; Weisz et al., 1985) the permeability (P) of nodules to gas exchange decreases. The decrease in P limits O_2 entry into the nodule, decreases respiratory production of energy and reductant needed to support N_2 fixation, and protects the O_2 labile dinitrogenase enzyme complex from inactivation (Layzell and Hunt, 1990).

A considerable amount of research has been devoted to the effects of water deficits on dinitrogenase activity and bulk nodule water potential. Pankhurst and Sprent (1975a) were the first to report that the decrease in dinitrogenase activity during nodule desiccation was associated with decreases in total bulk nodule water potential ($\bar{\Psi}_{tot}$, MPa), nodule respiration, and P. They further demonstrated that respiration and dinitrogenase activity from isolated bacteroids and nodule breis were independent of nodule desiccation treatments

except under conditions of extreme stress ($\bar{\Psi}_{tot}$<-1.3 MPa). The authors concluded that the inhibition of dinitrogenase activity due to nodule desiccation was dependent upon changes in nodule structure and not upon the biochemical competency of the bacteroids. However, their results were based upon the desiccation of detached nodules, and detachment itself causes a rapid decrease in dinitrogenase activity, nodule respiration, and P (Ralston and Imsande, 1982; Sung et al., 1991).

The effect of water deficits on nodule activity has also been determined on intact plants. Drought treatments extended over a period of 8 to 10 days result in a progressive decrease in dinitrogenase activity (Durand et al., 1987; Djekoun and Planchon, 1991; Guerin et al., 1990, 1991; Sall and Sinclair, 1991; Weisz et al., 1985), $\bar{\Psi}_{tot}$ (Durand et al., 1987; Djekoun and Planchon, 1991; Guerin et al., 1990, 1991), bulk nodule osmotic potential ($\bar{\Psi}_{\pi}$, MPa) (Sall and Sinclair, 1991), and P (Durand et al., 1987; Weisz et al., 1985). Increased O_2 concentration surrounding roots and nodules partially restores dinitrogenase activity in drought-stressed plants (Guerin et al., 1991; Weisz et al., 1985). This response indicates that the permeability of the nodule to O_2 may be insufficient to meet maximal respiratory and dinitrogenase potential.

In addition to decreased P, prolonged water deficits lead to other changes within the plant which may interact with P and the response of dinitrogenase activity. Proteolytic activity increases and protein and leghemoglobin decrease in nodules from plants drought-stressed 6 days or more (Guerin et al., 1990). A rapid imposition of water deficit treatments may avoid complications arising from

treatments extending over several days and help in understanding the effect of water deficits on dinitrogenase activity and P. Hormonal changes also occur during drought-stress, and in particular abscisic acid (AbA) concentration increases (Zhang and Davies, 1987). Accumulation of AbA may interact with carbohydrate partitioning (Clifford et al., 1986) and alter components of water potential (Robertson et al., 1990). The effect of exogenous AbA application to nodules on dinitrogenase activity and P has not been determined.

An additional factor interacting with the response of nodule activity to water deficits is whether nodules respond directly to a desiccating environment or to a decrease in plant water status. Exposing nodules to desiccating environments decreases dinitrogenase activity in drought-stressed plants but has little effect in well-watered plants (Khanna-Chopra et al., 1984; Sall and Sinclair, 1991). Using aeroponically grown plants, it is possible to subject the nodulated root crown to dry air or humidified air in well-watered or water-stressed plants. The different combinations of these treatments should allow the separation of local nodule environment from plant water status.

Physiological mechanisms of P adjustment are not well understood. Pankhurst and Sprent (1975b) concluded that morphological alterations in the nodule could account for P decreases. They found that nodule lenticels collapsed during desiccation, and hypothesized that the turgor loss of cells surrounding the lenticels would restrict O_2 diffusion into the nodule. Similarly, Ralston and Imsande (1982) theorized that the rapid loss of dinitrogenase activity following nodule detachment was due to turgor related collapse of cells composing the

lenticels, decreasing P. However, recent photomicrographs indicate the presence of large intercellular air spaces between lenticels (Dakora and Atkins, 1990; Parsons and Day, 1990), and closure of lenticels would appear to have little effect on the diffusion pathway for gases.

Alternatively, P adjustment is believed to result from changes in the intercellular water content in the nodule cortex. An increase in intercellular water results in thickening the water-filled diffusion barrier, decreasing P (Figure 1-2; equation 1-1). Varying the components of nodule water potential could serve to regulate the movement of water between intercellular spaces (equation 1-3), and thereby control the thickness of a diffusion barrier.

If decreases in P are achieved through an increase in intercellular water as proposed, then P regulation under water deficit conditions presents a paradox: the apoplastic water content in the nodule cortex is predicted to increase while total water content of the nodule decreases. If the apoplastic osmotic potential is assumed to be negligible, then a decrease in $\bar{\Psi}_{tot}$ represents an increase in the hydrostatic tension (H_t, MPa) applied to the water in intercellular spaces (Nobel, 1991). An increase in apoplastic H_t will in turn cause a decrease in the radius (r, m) of pores that remain filled with water according to the relationship (Nobel, 1991):

$$r = (2\sigma \cos\alpha) / H_t \qquad (4\text{-}1)$$

where σ is the surface tension of water (7.20×10^{-8} MPa m at 25°C) and α is the contact angle of the water-pore interface. For wettable walls under tension, α approaches zero (i.e., $\cos\alpha = 1$). Therefore, conditions which cause $\bar{\Psi}_{tot}$ to

decrease would seemingly cause P to increase, in contrast to the observed response of decreased P.

One possibility to account for an increase in apoplastic water content of the nodule cortex while $\bar{\Psi}_{tot}$ decreases is that solutes are moved from the symplasm to the apoplasm. The movement of solutes into the cortical apoplast would result in: (1) decreasing the osmotic potential gradient between the symplast and apoplast, (2) a net flux of water into the apoplast (equation 1-3), and (3) decreasing H_t in intercellular spaces. The decrease in H_t of intercellular spaces allows an apoplastic, water-filled diffusion barrier to be maintained while $\bar{\Psi}_{tot}$ decreases in response to water deficits. If water deficit treatments lead to a decrease in $\bar{\Psi}_{tot}$, and if P is unaffected or decreases, then it is indirect evidence for a decrease in H_t, possibly by apoplastic solute accumulation.

The identification of apoplastic solutes in nodules has only recently been attempted (Streeter, 1992). In the cortex of soybean nodules, Streeter (1992) reported that the apoplastic concentration of ureides, and in particular allantoic acid, increased linearly as the time following detachment increased. Although a causal relationship between apoplastic ureides and P has not been established, nodule detachment results in a rapid decrease in P (Sung et al., 1991).

Bulk nodule ureides also increased in treatments which decreased phloem import into nodules such as decreased photoperiod (Walsh et al., 1989a), and NO_3^- application (Streeter, 1985) and these same treatments are associated with a decrease in P (Vessey et al., 1988; Walsh et al., 1987; Walsh et al., 1989a). Restricted phloem import was believed to increase ureide content in nodules by

limiting the amount of water available for export in the xylem (Walsh et al., 1989a).

Additionally, water deficits result in an increase in nodule ureides (Sheoran et al., 1981) which may be related to a decrease in phloem supply to nodules. Water deficit treatments may decrease phloem transport by decreasing xylem water potential in the leaf. When leaf xylem water potential is less than or equal to leaf phloem water potential, phloem transport ceases (Nobel, 1991). In addition to nodule ureide content, soluble sugars have been reported to increase during drought treatments (e.g., Fellows et al., 1987), and the ratio of symplastic to apoplastic concentrations of sucrose and ureides have been proposed to account for changes in P (Hunt et al., 1990). Quantification of nodule solutes in response to rapid water deficit treatments and their effect on nodule water potential, P, and dinitrogenase activity have not been determined.

The amount of solute required to cause a change in P is dependent upon the water release properties of the nodule (equation 3-8). Results from pressure-volume analysis in Chapter 3 indicated that nodules desiccating while detached from the plant were highly elastic and had water release properties favorable for the regulation of P by an osmotic mechanism. However, the water release properties of nodules desiccating while attached to the plant have not been evaluated and may be different from detached nodules.

The first objective of experiments in this chapter was to manipulate components of bulk nodule water potential and determine the effect on dinitrogenase activity, P, and nodule respiration. By imposing rapid water deficit

treatments, nodule water potential may be altered and the effect on P, dinitrogenase activity, and nodule respiration determined without complications arising from several days of drought treatment (Guerin et al., 1990). The second objective was to characterize the effect of water deficit treatments on nodule solutes and the role they may have in P regulation. Soluble sugars and ureides have been implicated in P regulation by an osmotic mechanism (Hunt et al., 1990), and solutes may be important in varying nodule water potential, H_t, and the thickness of a water-filled diffusion barrier. The third objective was to evaluate the water release properties of nodules desiccating on the plant, and to determine if water release properties were favorable for P regulation by an osmotic mechanism.

Materials and Methods

Acetylene Reduction, Nodule Permeability, and Water Potential Determination

Soybean plants (cv. Biloxi) were grown in the greenhouse using the aeroponic system described in Chapter 2. When plants had developed three to four fully expanded leaves they were brought to the laboratory 2 days prior to treatment initiation and placed under high intensity lamps delivering 2300 μmol m^{-2} s^{-1} PPFD at plant height. The photoperiod was set for 16 h beginning at 0600.

The evening before an experiment began, a plant was transferred from an aeroponic chamber to a flow-through stainless steel assay chamber (Weisz and Sinclair, 1987a). The top 9 cm of the root was passed through a bored hole in the assay chamber. The stem and roots were sealed at the top and bottom of the

chamber, respectively, by passing the plant through bored stoppers, sealing the stem and roots with putty and silicon grease, and fitting the stopper assembly into the assay chamber. The lower portion of the root system was maintained in a separate, aerated nutrient reservoir. Humidified air was passed through the assay chamber at 0.33 ml s^{-1}.

The following morning at 0700 an initial measurement of acetylene reduction activity (ARA) and P was made. A 10% (v/v) C_2H_2 in air mixture was introduced into the assay chamber at a high volumetric flow rate. The dinitrogenase dependent reaction converting C_2H_2 into C_2H_4 (Hardy et al., 1968) was measured every 4 s throughout the following 2 min period. Steady-state ARA was determined directly using standard gas chromatography (Hardy et al., 1968), and P was calculated based upon the time required to reach steady-state ARA (Weisz and Sinclair, 1988a).

At 0800 (time=0, Figures 4-1 and 4-2), one of five different treatments were imposed: (1) Control; (2) AbA, 2 x 10^{-3} M (±) abscisic acid (AbA) was sprayed on nodules and roots in the assay chamber; (3) Dry Air, dehumidified air (dew point 4°C) was passed through the assay chamber at 0.33 ml s^{-1}; (4) PEG, nutrient solution was replaced with -1.0 MPa solution of polyethylene glycol (average molecular weight-6000); and (5) PEG + Dry Air, nutrient solution was replaced with -1.0 MPa PEG solution and dehumidified air was passed through the assay chamber. The AbA treatment was imposed to assess if gross hormonal changes which occur during water deficit treatments, such as AbA accumulation (Zhang and Davies, 1987), have a direct effect on dinitrogenase activity and P.

Combining the dry air and PEG treatments in different combinations were used to separate the effects of local nodule environment from plant water status. At 0.5, 2, 4, and 7 h following treatment initiation, ARA and P were measured.

At the end of the experimental period, four nodules from 3 to 4 mm in diameter were removed from the upper root system and placed in an osmometer (Model 5500, Wescor, Logan, UT) to determine $\bar{\Psi}_{tot}$. After $\bar{\Psi}_{tot}$ determination, nodules were frozen overnight and the sap was expressed onto filter discs to determine bulk nodule osmotic potential ($\bar{\Psi}_{\pi}$). Bulk nodule pressure potential was calculated as the difference between $\bar{\Psi}_{tot}$ and $\bar{\Psi}_{\pi}$. The remainder of the nodules in the assay chamber were removed from the plant, weighed, dried at 80°C, and reweighed. Each treatment was replicated six times.

Nodulated Root Respiration

Nodulated root respiration of intact plants was monitored in separate experiments using the stainless steel assay chamber described for measuring ARA. Plants were sealed in the assay chamber the night before an experiment began, and the next morning an initial respiration measurement was made at 0700 (time=-1, Figure 4-5). Flow rate through the assay chamber was increased from 0.33 to 1.8 ml s^{-1}, and the gas stream exiting the assay chamber was passed through an infra-red gas analyzer (Model 6200, Licor Inc., Lincoln, NE). The increase in CO_2 due to respiration was determined by the difference in CO_2 concentration entering and exiting the assay chamber.

One hour after the initial respiration measurement, one of three treatments were imposed: (1) Control; (2) PEG, -1.0 MPa PEG replaced nutrient solution;

(3) PEG + Dry Air, -1.0 MPa PEG replaced the nutrient solution and dry air (dew point 4°C) replaced the humidified gas flow through the assay chamber. Nodulated root respiration measurements were made at 0.5, 2, 4, and 7 h after treatment initiation. At the end of the experiments, nodules were removed from the root and the attached root system was resealed into the assay chamber. Following an 1 h equilibration period, bare-root respiration was determined, and nodule respiration was calculated as the difference between nodulated root respiration and bare-root respiration. Dry weights of root and nodules contained in the assay chamber were determined after drying at 80°C for 72 h.

Solute and Starch Analysis of Nodules

Dried nodules from plants in the ARA and P response experiments were ground with mortar and pestle and analyzed for total nonstructural carbohydrates (TNC), soluble sugars, and ureides. For TNC analysis, a procedure similar to that of Upmeyer and Koller (1973) was followed. A 25-mg subsample of ground nodule tissue was placed in a 2.5 x 15 cm test tube. Five ml of deionized water, 10 ml of acetate buffer (pH=4.45), and 0.2 mg thymol were added to each test tube. The test tubes were placed in a boiling water bath for 20 minutes, and after cooling, 2 units of amyloglucosidase (from Aspergillus niger, E.C. 3.2.1.3, Sigma Chemical Co., St. Louis, MO) were added. The samples were incubated in a water bath at 55°C for 48 h.

At the end of the incubation period, the samples were filtered, the residue was washed three times, and the samples brought to 100-ml volume. Approximately 20 ml of the extract was stored in plastic vials at -20°C until

analysis. The phenol-H_2SO_4 colorimetric procedure, described in Chapter 3, was used to quantify TNC in the nodule extract. Nodule starch was calculated as the difference between TNC and soluble sugars, multiplied by 0.9 (Upmeyer and Koller, 1973).

Soluble sugars and ureides were extracted in 10-ml of deionized water from 35 mg of dried and ground nodule material. After incubation in a water bath at 100°C for 2.5 h, the sample was filtered, and the residue was washed three times with deionized water. The extract was brought to a 50-ml volume and 20 ml was stored in plastic vials at -20°C until analysis. Soluble sugars and ureides were analyzed by the colorimetric procedures described in Chapter 3.

Water Release Properties of Nodules From Intact Plants

Changes in the components of bulk nodule water potential were determined during a 7 h desiccation treatment of intact plants. Soybean plants were sealed into the stainless steel assay chamber the night before an experiment began. The following morning at 0700 (time=-1 h), six nodules from 3 to 4 mm in diameter were removed from the portion of the root system contained in the assay chamber. Half of the nodules were used to determine $\bar{\Psi}_{tot}$, $\bar{\Psi}_{\pi}$, and $\bar{\Psi}_{p}$ as described previously. The remaining nodules were weighed to determine fresh weight (FW, g) and, after drying at 80°C for 72 h, dry weight (DW, g). Nodules harvested at the initial time period were assumed to be turgid, and therefore the turgid weight (TW) was the same as the fresh weight. The fractional water content (FWC) of nodules was calculated as follows:

$$FWC = (FW - DW) / FW \qquad (4\text{-}2)$$

At 0800 h, two treatments were imposed: (1) Control; (2) -1.0 MPa PEG replaced nutrient solution. The components of bulk nodule water potential, FW, and DW were determined at 2, 4, and 7 h after treatments began. The FWC of nodules at the initial measurement (FWC_{int}) was used to estimate the TW of nodules at subsequent time periods:

$$TW = DW / (1 - FWC_{int}) \qquad (4\text{-}3)$$

Assuming that nodules at the initial harvest were completely turgid, the estimated TW values were used to calculate relative water content (RWC) at subsequent harvests (equation 3-1).

Results

Acetylene Reduction Activity, Nodule Permeability, and Water Potential

Initial ARA ranged from 0.99 to 1.25 $mm^3\ s^{-1}\ gdw^{-1}$ (Table 4-1). These rates are similar to other reports of ARA on intact plants using flow-through systems which ranged from 0.67 (Weisz and Sinclair, 1987b) to 1.11 $mm^3\ s^{-1}\ gdw^{-1}$ (Parsons and Day, 1990). Reported values of ARA for excised nodulated roots in closed containers range from 0.097 (Djekon and Planchon, 1991) to 0.366 $mm^3\ s^{-1}\ gdw^{-1}$ (Kohl et al., 1991). The low ARA of excised nodulated roots indicates a significant error in measurement and may preclude understanding responses of ARA in intact plants.

Prior to treatment initiation, P ranged from 0.024 to 0.032 mm s^{-1} (Table 4-1). These P values are consistent with previous P determinations using the lag-phase (0.015 to 0.021 mm s^{-1}) (Dakora and Atkins, 1990; Weisz and Sinclair, 1987b) and CO_2 efflux (0.043 mm s^{-1}) (Minchin et al., 1986) techniques.

Table 4-1. Nodule characteristics, initial acetylene reduction activity (ARA), and initial nodule permeability (P) for plants used in the time course study of ARA and P response to water deficits. Values reported are the mean ± se (n=6).

Treatment†	Nodule Dry Weight	Nodule Number	Nodule Diameter	Initial‡ ARA	Initial‡ P
	-----g-----		----mm----	$mm^3\ s^{-1}\ gdw^{-1}$	$mm\ s^{-1} \times 10^3$
Control	0.14 ± 0.02	52 ± 11	2.5 ± 0.1	1.05 ± 0.08	29 ± 3
AbA	0.12 ± 0.01	38 ± 5	2.6 ± 0.1	0.99 ± 0.06	29 ± 2
Dry Air	0.11 ± 0.01	47 ± 7	2.4 ± 0.1	1.18 ± 0.04	32 ± 1
PEG	0.12 ± 0.02	59 ± 7	2.2 ± 0.1	1.25 ± 0.10	24 ± 1
PEG + Dry Air	0.11 ± 0.01	50 ± 6	2.3 ± 0.1	1.24 ± 0.11	28 ± 2

† Treatments were imposed for 7 h and consisted of the following: Control; AbA, 2×10^{-3} M abscisic acid was sprayed on nodules; Dry Air, dry air with a dew point of 4°C was passed over nodules at 20 ml min^{-1} in a well-watered plant; PEG, -1.0 MPa polyethylene glycol solution replaced nutrient solution; PEG + Dry Air, -1.0 MPa PEG solution replaced nutrient solution and dehumidified air was passed through the assay chamber.

‡ Initial measurements of ARA and P were before treatment imposition. To be consistent with units of P, units of ARA were expressed as $mm^3\ s^{-1}\ gdw^{-1}$. To convert $mm^3\ s^{-1}\ gdw^{-1}$ to $\mu mol\ h^{-1}\ gdw^{-1}$ (at 25°C), multiply by 147.

Peak ARA for the Control, AbA, and Dry Air treatments was unaffected during the 7-h exposure period (Figure 4-1). Replacing the nutrient solution with -1.0 MPa PEG resulted in severe leaf wilt within 0.5 to 1 h of treatment. The PEG treatment resulted in a sharp decrease in ARA from 90 to 64% of the initial rate between 4 and 7 h following treatment initiation. For the PEG + Dry Air treatment, ARA decreased linearly to 48% of the pretreatment rate by the end of the experiment.

Nodule permeability was unaffected by the Control, AbA, Dry Air, and PEG treatments (Figure 4-2). In contrast, P of the PEG + Dry Air treatment was significantly less than the Control from 2 to 7 h after treatments began, and was 72% of the initial P at the end of the experiment.

Although the Dry Air treatment had no effect on peak ARA, the kinetics of C_2H_4 production were altered during the 2 min C_2H_2 exposure period (Figure 4-3, Table 4-2). In the Control treatment, ARA peaked 40 to 60 s following the introduction of C_2H_2 into the assay chamber, and these rates were approximately the same during the remainder of the 2 min assay (Figure 4-3). Similar kinetics of C_2H_4 accumulation and peak ARA were also found throughout the 7-h experimental period for the Control treatment. However, in the Dry Air treatment, once ARA peaked there was a decline in ARA during the remainder of the 2 min measurement period (Figure 4-3). This response was evident within 30 minutes of the Dry Air treatment and increased in severity during the course of the experimental period (Table 4-2). The decline in ARA during the 2 min assay for the Dry Air treatment was not associated with P changes (Figure 4-2).

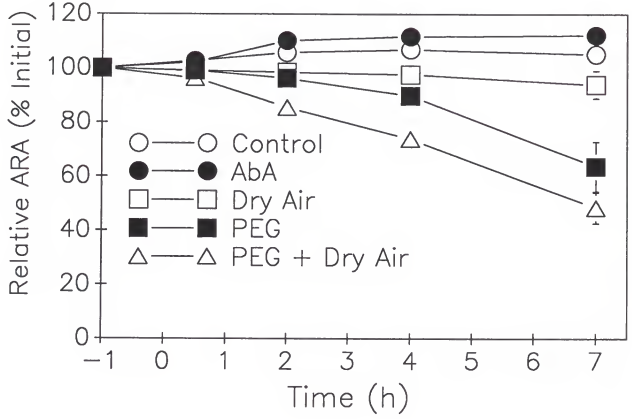

Figure 4-1. Relative acetylene reduction activity (ARA) versus time for five treatments imposed at time = 0. For treatment description and initial rates of ARA refer to Table 4-1. Values reported are the mean ± se (n=6).

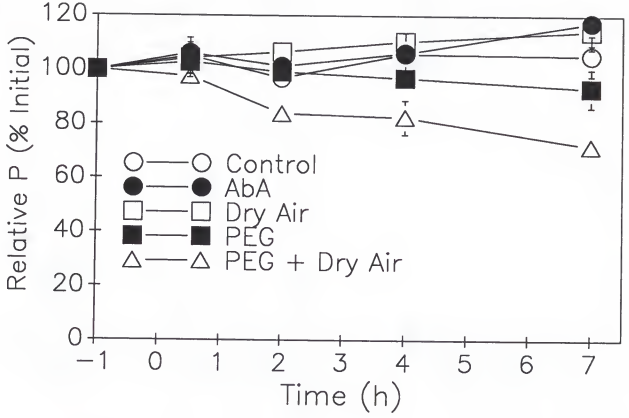

Figure 4-2. Relative nodule permeability (P) versus time for five treatments imposed at time=0. For treatment description and initial P refer to Table 4-1. Values reported are the mean ± se (n=6).

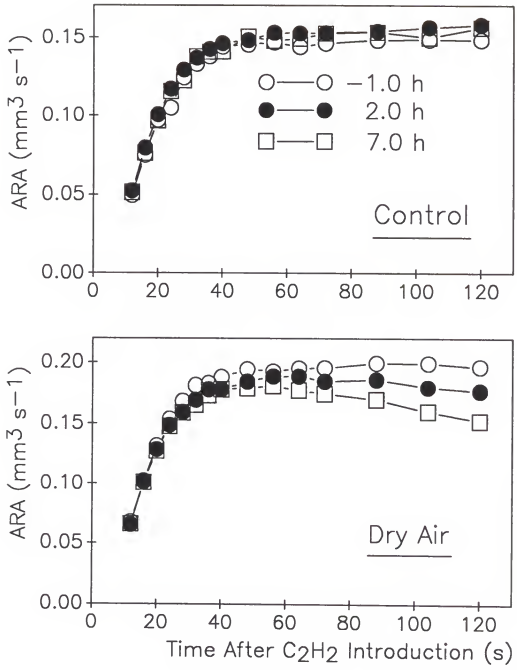

Figure 4-3. Time course of acetylene reduction activity (ARA) following the introduction of C_2H_2 at time=0 into a flow-through chamber. Measurements were made at -1, 2, and 7 hours after treatment initiation in Control plants and in well-watered plants with dry air passed over the nodules.

Table 4-2. Acetylene reduction activity (ARA) after 2 minutes of exposure to 10 % (v/v) C_2H_2, expressed as a percentage of the peak ARA. Values reported are the mean ± se (n=6).

	Time Following Treatment Initiation (h)				
Treatment†	-1.0	0.5	2.0	4.0	7.0
	----------% Peak ARA ----------				
Control	98.0 ± 1.2	98.1 ± 0.9	98.4 ± 0.8	98.3 ± 0.6	99.2 ± 0.3
AbA	98.4 ± 0.5	97.9 ± 1.6	98.6 ± 0.4	98.5 ± 0.7	99.1 ± 0.4
Dry Air	99.0 ± 0.6	95.3 ± 1.3	93.1 ± 1.4	89.5 ± 3.2	77.4 ± 5.6
PEG	99.3 ± 0.3	99.6 ± 0.3	99.1 ± 0.4	99.1 ± 0.5	98.6 ± 0.8
PEG + Dry Air	99.6 ± 0.2	96.1 ± 1.1	98.3 ± 0.7	97.2 ± 1.1	96.0 ± 1.5

† Treatments were imposed for 7 h and consisted of the following: Control; AbA, 2 x 10^{-3} M abscisic acid was sprayed on nodules; Dry Air, dry air with a dew point of 4°C was passed over nodules at 20 ml min^{-1} in a well-watered plant; PEG, -1.0 MPa polyethylene glycol solution replaced nutrient solution; PEG + Dry Air, -1.0 MPa PEG solution replaced nutrient solution and dehumidified air was passed through the assay chamber.

Control plants, with P similar to plants of the Dry Air treatment, exhibited no decline in ARA, even when the exposure period was extended to 10 min (data not shown).

The rapid decline in ARA of the Dry Air treatment (40 to 60 s, Figure 4-3) has not been described previously. Davey and Simpson (1990) found a decrease in the time required to cause a decline in ARA in nodules of subterranean clover (Trifolium subterraneum, L.) from 8 minutes in drought-stressed plants to 5 minutes in plants recovering from a drought. They attributed this response to an increase in P upon rewatering. However, CO_2 efflux from nodules in their study indicated no significant change in P during the recovery period, and data presented in Figures 4-1 and 4-2 fail to indicate a change in P or peak ARA associated with the Dry Air treatment (Figure 4-1, Figure 4-2).

A similar response to the decline in ARA was observed when nodules were exposed to chloroform vapor (Witty and Minchin, 1990). Anesthetics, such as chloroform, inhibit active ion transport systems across membranes (Kasamo, 1988) and increase permeability of membranes to ions (Jackson and St. John, 1984). It is not known if C_2H_2 causes a similar effect on ionic transport. However, a decrease in active ion transport and an increase in membrane permeability to ions would lead to an efflux of ions from the symplast to the apoplast. Increased ion concentration in the apoplast would result in water accumulation in the apoplast and a likely increase in the thickness of a water-filled diffusion barrier. Ultimately these events may lead to a decrease in P and ARA. There is no apparent explanation why exposure of nodules to dehumidified air should

decrease the time required to cause this response. In measurement systems using either low volumetric flow rates or closed systems, the decrease in ARA as the length of C_2H_2 exposure increases would appear as a decrease in ARA.

Exogenous AbA application to roots and nodules had no effect on ARA or P (Figures 4-1, 4-2). Applying a 0.05 MPa vacuum to roots and nodules after AbA application in order to draw AbA into the nodules was also without effect on ARA or P (data not shown). While exogenous AbA mimics the effect of drought treatment on $\bar{\Psi}_p$ in sunflower root tips (Robertson et al., 1990), there was no effect of exogenous AbA on the water relations of soybean nodules (Figure 4-4).

At the end of the experimental period, the components of bulk nodule water potential for the AbA, and Dry Air treatments were approximately the same as the Control treatment (Figure 4-4). Both the PEG and PEG + Dry Air treatments resulted in significant decreases in $\bar{\Psi}_{tot}$ and $\bar{\Psi}_p$ and a correspondingly large decrease in ARA (Figure 4-1). However, within the PEG and PEG + Dry Air treatments the decrease in ARA was not correlated with changes in $\bar{\Psi}_{tot}$, $\bar{\Psi}_\pi$, or $\bar{\Psi}_p$ (probability > 0.05, n=12, data not shown). Both the PEG and PEG + Dry Air treatments had similar effects on nodule water potential (Figure 4-4), but only the PEG + Dry Air treatment had significant effects on P (Figure 4-2).

Nodulated Root Respiration

Nodule respiration for the Control treatment at the end of the experiment was 3.05 $mm^3\ s^{-1}\ gdw^{-1}$ (Table 4-3) and within the range of reported nodule respiration rates of 1.63 (Witty et al., 1984) to 4.41 $mm^3\ s^{-1}\ gdw^{-1}$ (Sung et al.,

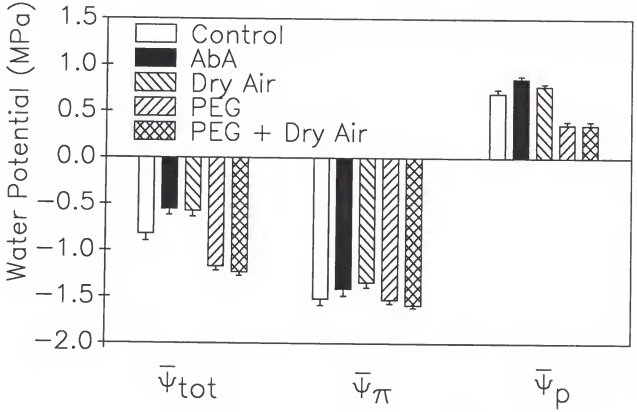

Figure 4-4. Total bulk nodule water potential ($\bar{\Psi}_{tot}$), osmotic potential ($\bar{\Psi}_{\pi}$), and pressure potential ($\bar{\Psi}_{p}$) response following 7 h of the indicated treatment. For treatment description refer to Table 4-1. Values reported are the mean and se (n=6).

Table 4-3. Nodule and root dry weights, nodule fractional water content (FWC), and the components of nodulated root respiration after 7 h of treatment. Values reported are the mean ± se (n=5).

Treatment†	Nodule Dry Weight	Root Dry Weight	FWC‡	Respiration§		
				Nodulated Root	Root	Nodule
	g			$mm^3\ CO_2\ s^{-1} gdw^{-1}$		
Control	0.18 ± 0.04	0.22 ± 0.04	0.743 ± 0.008	1.91 ± 0.35	0.90 ± 0.06	3.05 ± 0.07
PEG	0.21 ± 0.02	0.27 ± 0.03	0.704 ± 0.004	1.22 ± 0.09	0.59 ± 0.03	2.06 ± 0.24
PEG + Dry Air	0.18 ± 0.04	0.22 ± 0.05	0.698 ± 0.010	1.14 ± 0.17	0.52 ± 0.08	1.90 ± 0.26

† Treatments were imposed for 7 h and consisted of the following : Control; PEG, -1.0 MPa polyethylene glycol solution replaced nutrient solution; PEG + Dry Air, -1.0 MPa polyethylene glycol solution replaced nutrient solution and dehumidified air (dew point 4°C) was passed through assay chamber.

‡ FWC was calculated as: (nodule fresh weight - nodule dry weight) / nodule fresh weight.

§ Root respiration was determined following nodule removal and after 1 h equilibration period; nodule respiration was calculated as the difference between nodulated root respiration and root respiration. To be consistent with units of permeability, units of respiration were expressed as $mm^3\ s^{-1}\ gdw^{-1}$. To convert $mm^3\ s^{-1}\ gdw^{-1}$ to $\mu mol\ CO_2\ h^{-1}\ gdw^{-1}$ (at 25°C), multiply by 147.

1991). For the Control treatment, nodule respiration rates per unit weight were 3.4 times higher than root respiration rates per unit weight (Table 4-3). The higher respiratory rate of nodules may be attributed to the energy intensive nature of dinitrogenase activity (Burris, 1991).

Both root and nodule respiration rates at the end of the experiment were significantly less for the PEG and PEG + Dry Air treatments than the Control treatment (Table 4-3). However, the fraction of root respiration of the total nodulated root respiration was 0.47 for the Control, 0.48 for the PEG, and 0.46 for the PEG + Dry Air treatments. This indicates that root and nodule respiration declined to similar extents during the experimental period. Therefore, relative nodulated root respiration rates presented in Figure 4-5 reflect the change in nodule respiration since the fraction of nodule respiration to total nodulated root respiration was unaffected by treatment (Table 4-3).

Relative respiration rates of the Control treatment were constant throughout the experiment (Figure 4-5). Similar results were found for O_2 uptake by nodules in well-watered plants (Weisz and Sinclair, 1987a). In contrast to the Control treatment, there was a linear decrease in relative respiration from 0.5 to 7 h after treatments began for the PEG and PEG + Dry Air treatments. The decline in relative respiration for the PEG and PEG + Dry Air treatments (Figure 4-5) was similar to the decline in relative ARA (Figure 4-1) in terms of both the rate and relative magnitude of the decrease.

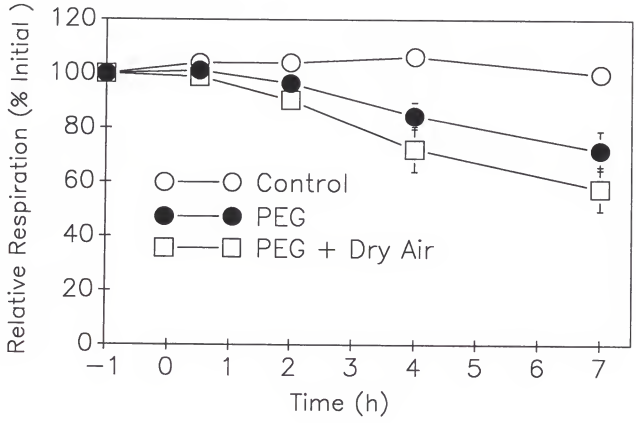

Figure 4-5. Relative nodule respiration versus time for three treatments imposed at time=0. For treatment description and initial rates of respiration refer to Table 4-3. Values reported are the mean ± se (n=5).

Solute and Starch Analysis of Nodules

In general, treatments which affected ARA (Figure 4-1) also had an effect on nodule soluble sugar, starch, and ureides (Table 4-4). Soluble sugar, starch, and ureide content of nodules were similar after 7 h of Control, AbA, and Dry Air treatments (Table 4-4). In contrast, soluble sugars in the PEG and PEG + Dry Air treatments decreased to 82 and 79% of the Control treatment, respectively. There was a greater decrease in nodule starch than soluble sugar content for the PEG and PEG + Dry Air treatments, amounting to 69 and 54% of the Control treatment, respectively.

Nodule ureide content of the PEG and PEG + Dry Air treatments increased after 7 h of treatment to 153 and 118% of the Control treatment, respectively. In the PEG and PEG + Dry Air treatments, severe leaf wilt was evident within 0.5 to 1 hour of treatment. Leaf wilting indicates a decrease in leaf water potential and, consequently, a decrease in xylem water potential and phloem export from leaves. The decrease in phloem transport associated with water deficits would decrease both the amount of water/photosynthate entering the nodule and the amount of water available to export ureides from the nodule. The greater nodule ureide content of the PEG treatment compared to the PEG + Dry Air treatment (Table 4-4) may have been due to higher rates of N_2 fixation during most of the treatment period (Figure 4-1) while having similar amounts of water available for ureide export.

Table 4-4. Soluble sugar, starch, and ureide content of nodules after 7 h of the indicated treatment. Values are the mean ± se (n=6)

Treatment†	Soluble Sugar	Starch	Ureide
	---------- mg gdw^{-1} ----------		μmol gdw^{-1}
Control	112 ± 1	61 ± 1	11.8 ± 0.7
AbA	106 ± 2	57 ± 6	11.8 ± 0.4
Dry Air	103 ± 2	54 ± 1	11.0 ± 1.0
PEG	92 ± 5	42 ± 6	18.1 ± 1.7
PEG + Dry Air	88 ± 2	33 ± 7	13.9 ± 1.0

† Treatments were imposed for 7 h and consisted of the following: Control; AbA, 2 x 10^{-3} M abscisic acid was sprayed on nodules; Dry Air, dry air with a dew point of 4°C was passed over nodules at 20 ml min^{-1} in a well-watered plant; PEG, -1.0 MPa polyethylene glycol solution replaced nutrient solution; PEG + Dry Air, -1.0 MPa PEG solution replaced nutrient solution and dehumidified air was passed through the assay chamber.

Water Release Properties of Nodules

There was a small, significant decrease in $\bar{\Psi}_{tot}$, $\bar{\Psi}_{\pi}$, and $\bar{\Psi}_{p}$ for the Control treatment by the end of the experiment (probability <0.05 for T-test) (Figure 4-6). For the PEG treatment, $\bar{\Psi}_{tot}$, $\bar{\Psi}_{\pi}$, and $\bar{\Psi}_{p}$ were significantly less than the Control treatment at each time period after treatments were imposed (Figure 4-6). There was also a faster decrease in RWC for the PEG treatment than the Control treatment over the course of the experiment (Figure 4-7). By the end of the experiment, RWC was 0.90 for the Control treatment and 0.71 for the PEG treatment.

Expressing $\bar{\Psi}_{p}$ as a function of RWC resulted in a linear relationship with a slope of 1.08 $\pm$ 0.14 (Figure 4-8). The bulk modulus of elasticity ($\bar{\epsilon}$, MPa) may be calculated from the product of the slope and the symplastic water content (equation 3-7). Assuming a fractional symplastic water content of 0.72 for nodules (Chapter 3), $\bar{\epsilon}$ was 0.78 $\pm$ 0.10 MPa which is substantially less than values of $\bar{\epsilon}$ for leaves ranging from 8 (Kubiske and Abrams, 1991) to 150 MPa (Campbell et al., 1979). However, the value of $\bar{\epsilon}$ for nodules desiccating on the plant was similar to both the $\bar{\epsilon}$ of nodules desiccating while detached from the plant (1.25 MPa, Chapter 3) and of palm stem (0.34 to 1.16 MPa, Holbrook and Sinclair, 1992). The low value of $\bar{\epsilon}$ in these tissues indicates a high degree of elasticity.

The relationship between $\bar{\Psi}_{tot}$ and RWC was linear with a slope of 2.72 MPa (Figure 4-9). The inverse of this slope defines the tissue capacitance (C_j, MPa^{-1}, equation 3-9) and has a value of 0.37 MPa^{-1}. Nodules desiccating on the plant had a C_j similar to that determined on nodules desiccating while detached

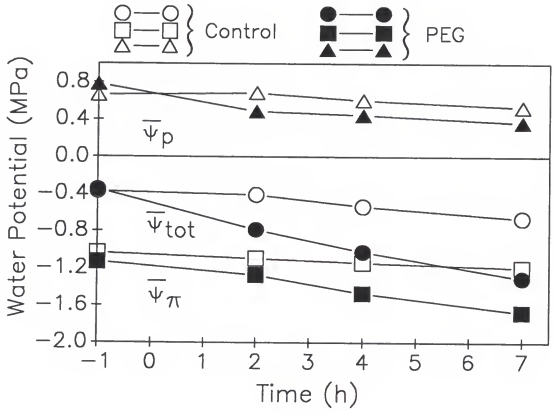

Figure 4-6. Total bulk nodule water potential ($\bar{\Psi}_{tot}$), osmotic potential ($\bar{\Psi}_\pi$), and pressure potential ($\bar{\Psi}_p$) versus time for control treatment and plants exposed to -1.0 MPa polyethylene glycol solution at time=0. Values reported are the mean (n=6). Standard error bars were smaller than figure symbols and averaged 0.05 MPa.

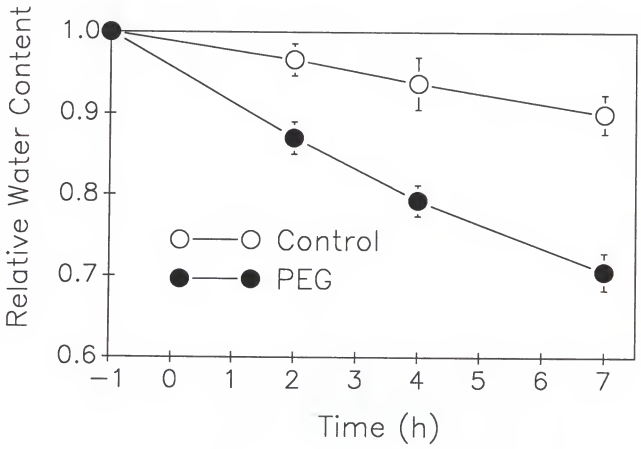

Figure 4-7. Relative water content of nodules versus time for Control plants and plants exposed to -1.0 MPa polyethylene glycol solution at time=0. Values reported are the mean ± se (n=6).

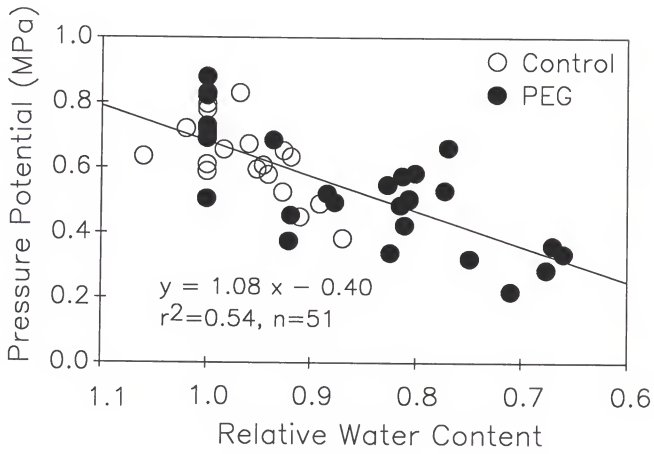

Figure 4-8. Bulk nodule pressure potential versus relative water content for Control plants and plants exposed to -1.0 MPa polyethylene glycol solution.

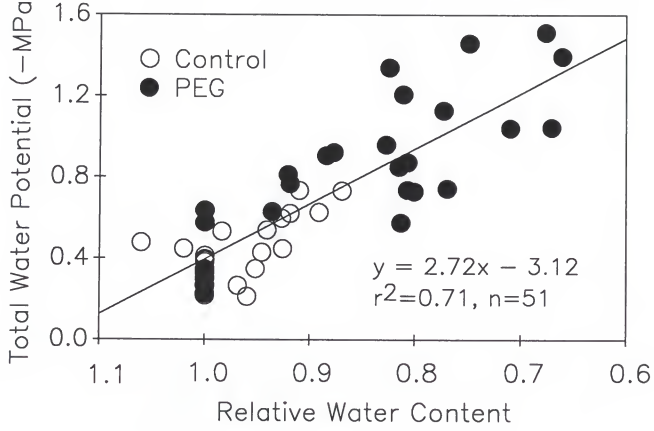

Figure 4-9. Total bulk nodule water potential versus relative water content for Control plants and plants exposed to -1.0 MPa polyethylene glycol solution.

from the plant (0.29 to 0.42 MPa^{-1}, Chapter 3). These values of C_j are an order of magnitude higher than calculated values of C_j (equation 3-10) from Quercus (Kubiske and Abrams, 1991) and soybean (Zur et al., 1981) leaves and similar to the C_j of palm stem (0.12 to 0.33 MPa^{-1}, Holbrook and Sinclair, 1992). The high C_j of palm stem functions to release water from the tissue when transpiration demand exceeds the supply from the soil.

Discussion

The effect of plant water deficits on P in these experiments differed from previous reports (e.g., Durand et al., 1987; Weisz et al., 1985) in that changes in dinitrogenase activity were not necessarily coupled to changes in P. This was evident in comparing the effect of the PEG treatment after 7 h on ARA (64% of the initial ARA, Figure 4-1) with P (93% of the initial P, Figure 4-2). In experiments where P decreased in response to drought, water deficits developed over a period of several days in either potted (Durand et al., 1987) or field-grown (Weisz et al., 1985) plants. The rapid imposition of water deficits in the aeroponic plants of these experiments may not have allowed sufficient time for P adjustment. The observed uncoupling of ARA and P is important because it demonstrates that P is not dependent directly on dinitrogenase activity.

Changes in the components of bulk nodule water potential did not have a direct effect on P. If there had been a direct effect on P then the response of P to the PEG and PEG + Dry Air treatments (Figure 4-2) would have been the same because the treatment effect on $\bar{\Psi}_{tot}$, $\bar{\Psi}_{\pi}$, and $\bar{\Psi}_p$ was similar (Figure 4-4). Hunt et al. (1990) reported an increase in $\bar{\Psi}_{\pi}$ under conditions of low P and

associated this response with a decrease in nodule sucrose concentration. However, results from Chapter 2 indicate that $\bar{\Psi}_{\pi}$ was similar in well-watered plants differing in P, and results from this chapter and Chapter 3 do not indicate a role for soluble sugars in P regulation at the bulk nodule level.

Comparing the effect of the Dry Air, PEG, and PEG + Dry Air treatments indicates that plant water status had a greater effect than the surrounding nodule environment on ARA (Figure 4-1), P (Figure 4-2), and nodule water relations (Figure 4-4). Similar conclusions were drawn from experiments comparing ARA of plants watered by a subsurface system with nodules in dry soil versus ARA of plants under water deficits with nodules in dry soil (Khanna-Chopra, 1984; Sall and Sinclair, 1991). However, combining a desiccating environment around root nodules with a water deficit treatment increased the severity of the response on ARA (Figure 4-1) and P (Figure 4-2) above that of the water deficit treatment alone.

The presence of solutes in the apoplast appears to be important with regards to P regulation during water deficits. If apoplastic solutes were negligible, then the decrease in $\bar{\Psi}_{tot}$ for the PEG and PEG + Dry Air treatments would result in both an increase in the hydrostatic tension (H_t) of water in the intercellular spaces and a decrease in the radius of pores remaining filled with water (equation 4-1). Assuming no apoplastic solutes, pore radii calculated to be filled with water for the Control, PEG, and PEG + Dry Air treatments based on H_t after 7 h of treatment were 0.18 $\pm$ 0.01, 0.12 $\pm$ 0.01, and 0.11 $\pm$ 0.00 μm (equation 4-1). A decrease in the radius of pores filled with water for the PEG

and PEG + Dry Air treatments would decrease the thickness of the diffusion barrier and increase P (equation 1-1). However, P for the PEG treatment remained constant and P for the PEG + Dry Air treatment decreased over the experimental period (Figure 4-2). This indicates that intercellular water content in the diffusion barrier may have remained constant or increased (equation 1-1) for the PEG and PEG + Dry Air treatments, respectively. The discrepancy between the calculated response of pore radii and the observed response of P may be reconciled if solutes accumulated in the apoplast, decreasing H_t.

Additionally, the water release properties of the nodule were favorable for P regulation by an osmotic mechanism. The low $\bar{\varepsilon}$ (0.78 MPa) may enable small amounts of solute movement from the symplast to apoplast to have large effects on intercellular water content (equation 3-8), and the high C_j (0.37 MPa^{-1}) of the nodule may enable the tissue to release relatively large amounts of water to the apoplast with only minor effects on the total water potential of the tissue (equation 3-9).

Although changes in components of bulk nodule water potential were not associated with P changes, this does not preclude the possibility that specific tissues within the nodule regulate diffusion barrier thickness by an osmotic mechanism. The accumulation of nodule ureides observed in this study in response to water deficits may function osmotically in P regulation. The increase in apoplastic ureide concentration following nodule detachment (Streeter, 1992) was associated with a decrease in dinitrogenase activity and P (Sung et al., 1991). Apoplastic ureides would decrease H_t and cause an increase in the radii of

intercellular spaces in the nodule cortex filled with water (equation 4-1). However, it remains to be determined if ureides accumulate apoplastically in response to water deficits, or if ureides accumulate symplastically in well-watered plants in response to 10 kPa O_2 (Chapter 3).

CHAPTER 5
DINITROGENASE ACTIVITY AND NODULE PERMEABILITY RESPONSE TO PHLOEM PRESSURIZATION IN SOYBEAN

Introduction

Dinitrogen fixation rates in soybean nodules are quantitatively linked to the permeability (P) of the nodule to O_2 diffusion (Hunt et al., 1987; Weisz and Sinclair, 1987b). Treatments which disrupt the phloem supply to nodules result in rapid decreases in both P and dinitrogenase activity. Dinitrogenase activity decreases by 50% within 5 minutes of nodule detachment (Sung et al., 1991) and within 2 h of stem girdling (Walsh et al., 1987). Other treatments which disrupt phloem transport into nodules, such as NO_3^- addition to nodules (Vessey et al., 1988) and decreased photoperiod (Walsh et al., 1987), result in decreases in dinitrogenase activity and P.

The phloem is the primary source of water for soybean nodules (Walsh et al., 1989a), and decreases in phloem transport restrict the amount of water that is available to export ureides from the nodule. Therefore, conditions which decrease phloem transport result in an increase in nodule ureides. In addition to stem girdling (Walsh et al., 1987), nodule detachment (Ralston and Imsande, 1982; Sung et al., 1991), NO_3^- addition (Vessey et al., 1988), and decreased photoperiod (Walsh et al., 1987), water deficit treatments decrease phloem supply

to nodules (Nobel, 1991) and result in an increase in nodule ureides (Chapter 4, Sheoran et al., 1981).

Nodule ureides may function osmotically to regulate P. Upon nodule detachment, P decreases rapidly (Sung et al., 1991) and there is an increase in the apoplastic concentration of ureides located in the nodule cortex (Streeter, 1992). An increase in the apoplastic concentration of solutes would fill intercellular spaces with water increasing the thickness of a water-filled diffusion barrier (equation 1-6), and decreasing P (equation 1-1). Other treatments, mentioned previously, which disrupt phloem transport are associated with both an increase in nodule ureide content and a decrease in P.

The relationship between phloem transport and P is not well understood. Ralston and Imsande (1982) proposed that severing phloem connections by nodule detachment caused a turgor-related collapse of cells surrounding the nodule lenticels. Lenticel closure would thereby restrict O_2 diffusion into nodules. A similar hypothesis was presented by Pankhurst and Sprent (1975b) to explain the decrease in P associated with nodule desiccation. However, recent photomicrographs (Dakora and Atkins, 1991; Parsons and Day, 1990) indicate that there are abundant intercellular air spaces between lenticels, and that closure of lenticels would not have a significant impact on the gas diffusion pathway.

Walsh et al. (1987) interrupted phloem transport by stem chilling and girdling, leaf removal, and various photoperiod treatments. The response of dinitrogenase activity, nodule respiration, and the carbohydrate content of various plant parts indicated that dinitrogenase activity was dependent upon a continual

phloem supply, and that nodule starch reserves were ineffective in providing soluble carbohydrates to nodules following phloem interruption. Walsh et al. (1987) concluded that a continual phloem supply was necessary to support dinitrogenase activity because of the carbohydrate dependency of the nodules upon phloem supply.

An alternative interpretation of the results of Walsh et al. (1987) is that a continual phloem supply was needed to provide the nodule with sufficient water to export ureides from nodule in the xylem. Accordingly, if nodules were supplied with water through the phloem with a carbohydrate free source, then ureide export in the xylem could continue upon phloem interruption. Continued ureide export would prevent both an accumulation of ureides and a proposed decrease in P due to apoplastic ureide accumulation.

In experiments described in this chapter, a solution, with and without sucrose, was placed around the exposed secondary phloem at a stem girdle site. The solution was forced through the phloem tissue to the nodules by pressurizing the solution with N_2 gas.

The first objective of this experiment was to develop a technique to provide nodules with carbohydrates and water through the exposed phloem tissue of a stem girdle. Such a technique would provide a means to study carbohydrate, hormone, and inhibitor effects on nodule metabolism. The second objective was to separate the effects of phloem water supply from phloem carbohydrate supply on P and dinitrogenase activity.

Materials and Methods

Plant Growth Conditions

Soybean plants were grown aeroponically in the greenhouse as described in Chapter 2. When plants had developed 3 to 4 fully expanded leaves, they were brought to the laboratory. In the laboratory, plants were kept for 2 days prior to treatment under high intensity lights delivering 2300 μmol m^{-2} s^{-1} PPFD at plant height. High intensity lights were set on 16 h photoperiods.

The evening before a measurement began the upper portion of the nodulated root system was sealed into the flow through assay chamber described in Chapter 4. The lower portion of the root system remained in aerated nutrient solution. Humidified air was passed through the assay chamber at 0.33 ml s^{-1}. The following morning at 0700 h, an initial measurement of acetylene reduction activity (ARA) (Hardy et al., 1968) and P (Weisz and Sinclair, 1988a) was made using the techniques described in Chapter 4.

Treatment Implementation

A potential problem associated with phloem manipulation experiments is blockage of the sieve elements (King and Zeevaart, 1974). Treating severed petioles (King and Zeevaart, 1974) and soybean pods (Fellows et al., 1978) with 20 mM EDTA prevented phloem blockage and allowed constant rates of phloem exudation up to 4 h after excision.

One hour after the initial measurement of ARA and P, one of four different treatments were imposed. (1) Control,[1] an intact plant was used throughout the experiment. (2) Detop + EDTA, upper portion of the plant was excised between the second and third nodes. A 1-cm region of outer bark was removed between the first and second nodes of the stem, exposing the phloem tissue. A modified pressure chamber (Figure 5-1) was sealed around the girdled region of the plant stem, and the pressure chamber was filled with 20 mM EDTA (pH 7.0). (3) Pressure + EDTA, same as the detop + EDTA treatment with the addition that the exposed phloem tissue of the stem girdle within the pressure chamber was pressurized to 0.1 MPa with N_2 gas. (4) Pressure + sucrose, same as the pressure + EDTA treatment except that 3 mM sucrose was added to the 20 mM EDTA and that a pressure of 0.2 MPa was applied. Preliminary experiments indicated an apparent phloem blockage with the sucrose solution at a pressure of 0.1 MPa, and this problem was seemingly avoided at a pressure of 0.2 MPa.

The pressure chamber was constructed from 1.91 cm (0.75 inch) standard pipe fittings (Figure 5-1). For the phloem pressurization treatments, a girdled stem was passed through a hole drilled in a 1.91-cm pipe cap. A 6-mm diameter tube was inserted through the bored hole of a #0 rubber stopper, and this assembly was positioned over the stem and below the girdled region. Removal of the tube from the stopper secured the stopper around the plant stem. A metal

[1]Plants used for controls in this experiment also served as controls for experiments in Chapter 4.

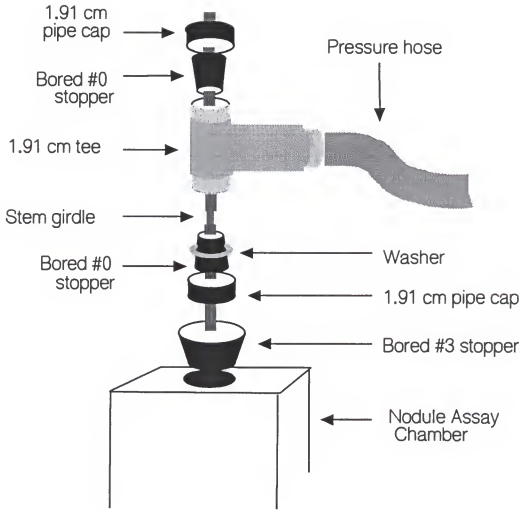

Figure 5-1. Expanded view of apparatus used to pressurize phloem tissue. A girdled stem was placed inside the shown chamber, filled with a 20 mM EDTA solution with or without 3 mM sucrose, and pressurized with N_2 gas. For details see text.

washer was centered around the circumference of the stopper to prevent the stem/stopper combination from turning when the pressure chamber was threaded onto the pipe cap. Next, the stem was inserted through the aligned openings of a tee fitting. The lower portion of the pressure chamber was sealed by threading the tee into the pipe cap. At the top of the pressure chamber, a 6-mm diameter tube was passed through the hole in a pipe cap and then through the bored hole in a #0 stopper. This assembly was lowered over the stem with the result that the stem passed through the 6 mm tube and that the stopper was inserted into the top of the tee fitting. Holding the tube stationary allowed the pipe cap to be threaded onto the tee fitting without damage to the plant stem. Upon removal of the 6-mm diameter tube, the stopper sealed the stem at the top of the pressure chamber. A 100-ml reservoir (not shown in Figure 5-1) was attached to the open end of the tee fitting, filled with 20 mM EDTA, and the chamber assembly was pressurized to 0.1 MPa with N_2 gas. The same procedure was followed when the phloem was pressurized with the sucrose solution except where noted previously. Approximately 5 minutes were required to seal the plant inside the pressure chamber.

Measurements of ARA and P were made at 0.5, 2, 4, and 7 h after the start of the treatments. At the end of the experiment, nodule fresh weight, dry weight, number, and diameter were determined for nodules contained within the assay chamber. The ARA and P results were expressed relative to values at time=-1 h within each treatment.

For treatments pressurizing the phloem, the stem was cut beneath the pressure chamber at the end of the experiment. The presence of exudate being forced through the vascular system from the lower end of the pressure chamber was used as a criterion to accept the experimental results. There was some leakage of the EDTA solution from where the bottom of chamber sealed around the plant stem. In those cases at the end of the treatment period when no exudate was observed or the chamber did not contain any EDTA solution, the experiment was discarded.

Phloem Pressurization and Phloem Movement

A plant was girdled and sealed into the pressure chamber as described in the previous section. A 20 mM EDTA solution (pH 7.0) containing 0.01% (w/v) of neutral red was added to the pressure chamber, and the system was sealed and pressurized to 0.1 MPa.

Nodules were removed from the upper root system periodically over the next 7 h. Free-hand nodule sections were made at right angles to the point of nodule attachment to the root and examined at 40x under a light microscope for the presence of stain. At the end of 7 h, free-hand cross sections of the stem were made at approximately 5 mm intervals beginning at the stem girdle site and proceeding towards the root. The fresh stem sections were examined at 40x under a light microscope for the presence of stain.

Results and Discussion

Before treatments were initiated, ARA ranged from 0.87 to 1.05 $mm^3\ s^{-1}\ gdw^{-1}$ and P ranged from 0.024 to 0.030 mm s^{-1} (Table 5-1). These values of ARA and P were similar to values of ARA and P reported in Chapter 4.

At 0.5 h after treatment initiation there was no difference in relative ARA between treatments (Figure 5-2). However, after 2 h, relative ARA for the pressure + EDTA treatment was significantly less than relative ARA for both the detop + EDTA and the pressure + sucrose treatments (probability < 0.05 for T-test). After 4 h of treatment, ARA had dropped to 18, 13, and 19% of the initial rates for the detop + EDTA, pressure + EDTA, and pressure + sucrose treatments, respectively. From 4 to 7 h after treatment initiation there was little further change in relative ARA.

Pressurization of the phloem with either the EDTA or sucrose solutions resulted in significant changes in P compared to the detop + EDTA treatment at 0.5, 2, and 7 h after the start of the treatment (Figure 5-3). After 0.5 h, P for the pressure + EDTA treatment was 107% of the initial rate compared with 96% for the detop + EDTA treatment (probability < 0.10 for T test). Relative P for the pressure + sucrose treatment was 91% after 2 h and 54% after 7 h compared to 64% after 2 h and 39% after 7 h for the detop + EDTA treatment (treatments significantly different at each time period, probability < 0.05 for T test).

The general lack of differences between treatments in relative ARA and P at the 4 and 7 h time periods may have been due to phloem blockage. With the phloem blocked, the pressure + EDTA and pressure + sucrose treatments were

Table 5-1. Nodule characteristics, initial acetylene reduction activity (ARA), and initial nodule permeability (P). Values reported are the mean ± se (n=6).

Treatment†	Nodule Dry Weight	Nodule Number	Nodule Diameter	Initial‡ ARA	Initial‡ P
	-----g-----		----mm----	$mm^3\ s^{-1}\ gdw^{-1}$	$mm\ s^{-1} \times 10^3$
Control	0.14 ± 0.02	52 ± 11	2.5 ± 0.1	1.05 ± 0.08	29 ± 3
Detop + EDTA	0.17 ± 0.02	113 ± 8	2.1 ± 0.1	0.93 ± 0.08	30 ± 4
Pressure + EDTA	0.14 ± 0.02	94 ± 6	2.1 ± 0.0	1.01 ± 0.06	25 ± 3
Pressure + sucrose	0.18 ± 0.01	76 ± 11	2.3 ± 0.1	0.87 ± 0.04	24 ± 1

† Treatments were imposed for 7 h and consisted of the following: control; detop + EDTA, upper portion of plant excised above second node, 1-cm stem bark removed between first and second nodes, and a 20 mM EDTA solution was placed around the girdle; pressure + EDTA, same as detop + EDTA with the addition that the phloem was pressurized at a pressure of 0.1 MPa; pressure + sucrose, same as pressure + EDTA treatment except that phloem tissue was pressurized at 0.2 MPa with a solution containing 3 mM sucrose.

‡ Initial measurements of ARA and P were before treatment imposition. To be consistent with units of P, units of ARA were expressed as $mm^3\ s^{-1}\ gdw^{-1}$. To convert $mm^3\ s^{-1}\ gdw^{-1}$ to $\mu mol\ h^{-1}\ gdw^{-1}$ (at 25°C), multiply by 147.

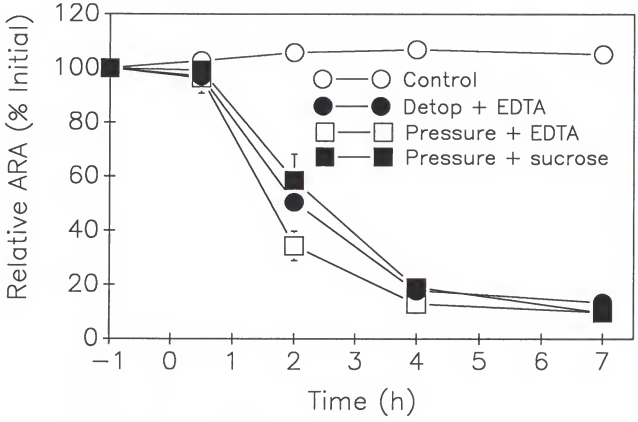

Figure 5-2. Relative acetylene reduction activity (ARA) versus time for four treatments imposed at time=0. For treatment description and initial rates of ARA refer to Table 5-1. Values reported are the mean ± se (n=6).

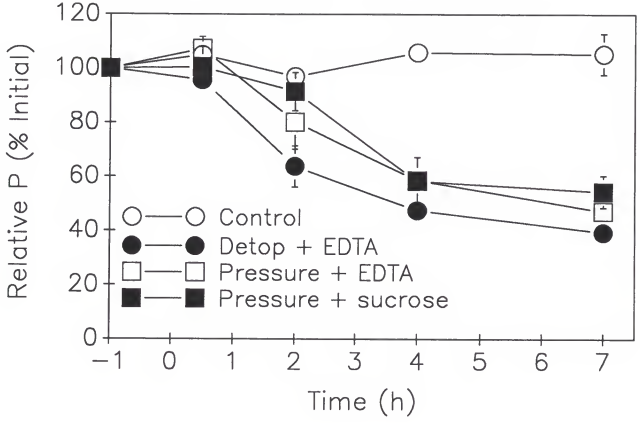

Figure 5-3. Relative nodule permeability (P) versus time for four treatments imposed at time=0. For treatment description and initial rates of P refer to Table 5-1. Values reported are the mean ± se (n=6).

equivalent to the detop + EDTA treatment. For the first 1 to 2 h of the phloem pressurization treatments, exudate flowed freely from the excised stem protruding above the pressure chamber. However, after this time period exudation decreased dramatically. There was no exudation from the excised stem of the detop + EDTA treatment. The decrease in exudation after 2 h of phloem pressurization was particularly evident when sucrose was included in the buffer solution. When the pressure was increased to 0.2 MPa for the pressure + sucrose treatment, exudation from the excised stem above the pressure chamber was comparable to exudation for the pressure + EDTA treatment. Stem exudation was also observed for the first 2 h when the phloem was pressurized with 0.01% neutral red-20 mM EDTA. However, no stain was observed in the exudate, and after 2 h the exudation rate decreased.

At the end of 7 h of phloem pressurization, exudate was observed from the stem severed beneath the pressure chamber for both of the phloem pressurization treatments. This indicates that the vascular system was not blocked. However, observed movement of a 0.01% neutral red-20 mM EDTA solution in the vascular system upon pressurization was restricted to the node in which the girdle was made. Cross sections of the stem closest to the girdle were stained uniformly. Further from the girdle, stain was present in both isolated xylem and phloem tissues. Additionally, there was no stain found in the root or nodule up to 7 h after the start of the phloem pressurization treatment. At phloem transport velocities of 0.78 cm min^{-1} (Housley and Fisher, 1977) stain should have reached nodules within 1 h.

The inability to detect stain in the nodules or in the phloem exudate is not understood. Neutral red might have been adsorbed in the vascular tissue near the girdle and essentially filtered out of the phloem exudate. Other vital stains may be more effective in detecting the movement of the buffer solution through the phloem. Labeled sucrose could also be added to the EDTA solution to detect respiratory utilization by the nodules of phloem derived carbohydrate.

A comparison of Figures 5-2 and 5-3 indicates that pressurization of the phloem with either the EDTA or sucrose solutions resulted in a separation of relative ARA and P at 2 h following treatment initiation. There was a 66% and 42% decrease in relative ARA at 2 h for the pressure + EDTA and pressure + sucrose treatments, respectively. These large decreases in relative ARA are compared with P decreases of only 20% for the pressure + EDTA treatment and 9% for the pressure + sucrose treatment. In contrast, the responses of P and ARA to the detop + EDTA treatment generally mirrored each other. For example at 2 h after treatment initiation, relative ARA for the detop + EDTA treatment was 50% and relative P was 64%.

Large initial decreases in relative ARA with small changes in relative P were also demonstrated when exogenous NH_3 was supplied to nodules (Appendix). The assimilation of exogenous NH_3 may have decreased the supply of nodule carbohydrates which would have otherwise been available to support dinitrogenase activity. Utilization of carbohydrate for NH_3 assimilation would decrease carbohydrate supply available to support dinitrogenase activity while

maintaining phloem import to nodules. In this regard, the NH_3 treatment had the same effect as the pressure + EDTA treatment on relative ARA and P.

There was no significant difference in P at 2 h between the pressure + sucrose treatment and the pressure + EDTA treatment (Figure 5-3). However, the addition of sucrose to the buffer pressurization treatment resulted in significantly higher relative ARA after 2 h than the pressure + EDTA treatment (probability < 0.05 for T-test) (Figure 5-2). This indicates that P may be mainly dependent upon water supplied through the phloem to the nodule, and that ARA may be dependent upon carbohydrate delivered through the phloem. A continual supply of nodule water may prevent nodule ureide accumulation (Walsh et al., 1989a) and an associated decrease in P.

The large decrease in relative ARA at 2 h for the pressure + sucrose treatment indicates that factors in addition to 3 mM sucrose solution were needed to support dinitrogenase activity (Figure 5-2). The EDTA solution removed divalent cations from the phloem, which may have been important in nodule function. Phloem exudate from soybean contains approximately 5 times the sucrose concentration used in this study (Housley and Fisher, 1977). A higher sucrose concentration pressurized through the phloem may allow continued dinitrogenase activity. However, preliminary experiments indicated that sucrose concentrations of 15 mM led to phloem blockage, as judged by the lack of exudate from the severed stem beneath the pressure chamber. In addition to higher sucrose concentration, phloem exudate contains organic and inorganic ions, amino acids, and growth regulators (Housley and Fisher, 1977; Layzell and

LaRue, 1982), and any of these factors may be needed to maintain dinitrogenase activity.

Pressurization of the secondary phloem in girdled plants was a novel method of separating the effects of phloem water supply from phloem carbohydrate supply on dinitrogenase activity and P. These experiments were consistent with the hypothesis that water derived from the phloem was required for maintenance of high P. A continual water supply from the phloem in girdled plants may have delayed a large P decrease by allowing the continued export of ureides in the xylem. In girdled plants without phloem pressurization ureides may accumulate apoplastically in the nodule cortex, increase the thickness of a water-filled diffusion barrier and decrease P.

CHAPTER 6
SUMMARY AND CONCLUSIONS

The permeability of the nodule to O_2 regulates the amount of O_2 entering the nodule. Nodule permeability is important for at least three reasons. First, permeability controls nodule respiration and the energy and reductant available for dinitrogen fixation (Layzell and Hunt, 1990). Second, permeability changes in response to environmental signals and stresses which have both physiological and agronomic significance (Weisz et al., 1985; Weisz and Sinclair 1987b, 1988b). Third, permeability decreases under conditions of decreased nodule carbohydrate supply (Walsh et al., 1987). Without a decrease in permeability, low nodule carbohydrates would lower respiratory consumption of O_2 and lead to an increase in nodule O_2 concentration. However, as a result of decreased permeability, O_2 concentration is maintained in the nanomolar range and dinitrogenase is protected from O_2 inactivation.

The experiments described in this dissertation focus on the possible mechanisms underlying nodule permeability changes. The central hypothesis tested was that permeability is controlled by the amount of water filling intercellular passages in the nodule cortex (Figure 1-2). Increasing intercellular water in the nodule cortex would increase the thickness of a water-filled diffusion barrier to O_2 and decrease permeability. Conversely, decreasing intercellular

water in the nodule cortex would decrease the thickness of the diffusion barrier and increase permeability. Results from Chapter 2 indicate that conditions of low nodule permeability result in a decrease in the air space content of nodules compared to controls (Table 2-2). The decrease in air space content under low permeability conditions indicates that water has displaced air pathways and may have increased the thickness of the diffusion barrier.

Under the hypothesis presented, the movement of water into and out of cells during permeability changes is due to a water potential gradient between the cortical symplast and apoplast. Experimentally, the components of bulk nodule water potential were measured by freeze-thaw and pressure-volume methodologies under conditions affecting nodule permeability.

In Chapter 2, roots and nodules of intact plants were exposed to 10 or 21 kPa O_2 for 24 h. The 10 kPa O_2 treatment caused an increase in nodule permeability of approximately 150% compared to the 21 kPa O_2 treatment (Weisz and Sinclair, 1987b). Bulk nodule osmotic potential ($\bar{\Psi}_\pi$) was not affected by O_2 treatment as measured by the freeze-thaw method (Table 2-1). In Chapter 3, roots and nodules were also exposed to 10 or 21 kPa O_2 treatments, and pressure-volume analysis was used to determine the effect of O_2 treatment on $\bar{\Psi}_{tot}$, and $\bar{\Psi}_p$ in addition to $\bar{\Psi}_\pi$. Results from Chapter 3 also indicated that there was no difference in $\bar{\Psi}_{tot}$, $\bar{\Psi}_\pi$, or $\bar{\Psi}_p$ between O_2 treatments (Figure 3-1, Table 3-1).

In Chapter 4, the effect of bulk nodule water potential components on permeability was evaluated differently from Chapters 2 and 3. Plants were exposed to water deficit treatments to alter the components of nodule water

potential, and this response was compared to changes in nodule permeability. It was demonstrated that a plant water deficit treatment combined with exposure of nodules to dry air resulted in decreases in dinitrogenase activity (Figure 4-1), nodule permeability (Figure 4-2), and components of bulk nodule water potential (Figure 4-4). A plant water deficit treatment in a non-desiccating environment also resulted in decreases in dinitrogenase activity (Figure 4-1) and components of bulk nodule water potential (Figure 4-4), but nodule permeability was unaffected by this treatment (Figure 4-2). This indicated that components of bulk nodule water potential did not have a controlling influence on nodule permeability.

Taken together, the results discussed above from Chapters 2, 3, and 4 did not indicate a role for bulk nodule water potential in controlling nodule permeability. This conclusion does not exclude the possibility that nodule permeability was controlled by changes in water potential in specific regions of the nodule. If water potential changes associated with permeability regulation were restricted to the inner cortex then this would represent water potential adjustment in only 3 to 5 cell layers in a nodule approximately 3 mm in diameter (Parsons and Day, 1990).

While the bulk nodule water potential measurements presented in Chapters 3 and 4 did not indicate a role in regulating permeability, the water release properties of nodules determined in Chapters 3 and 4 were consistent with permeability regulation by an osmotic mechanism. Specifically, the bulk modulus of elasticity ($\bar{\varepsilon}$) ranged from 1.25 MPa, for nodules desiccating while detached from the plant (Chapter 3), to 0.78 MPa, for nodules desiccating on the plant

(Chapter 4). These values of $\bar{\epsilon}$ are 1 to 2 orders of magnitude lower than $\bar{\epsilon}$ for leaf tissue (e.g., Campbell et al., 1979; Kubiske and Abrams, 1991) and indicate a high degree of elasticity for the bulk nodule. Additionally, the low $\bar{\epsilon}$ allows small changes in solute distribution to move relatively large amounts of water between the symplast and apoplast (equation 3-8).

Potential solutes responsible for osmotic changes were grouped into three classes, soluble sugars, free amino acids, and ureides, and analyzed in nodules after exposure to 10 or 21 kPa O_2 treatments (Chapter 3). After 24 h, dinitrogenase activity was similar for plants exposed to 10 and 21 kPa O_2 treatments. However, nodule permeability for the 10 kPa O_2 treatment increased to approximately 150% of the 21 kPa O_2 treatment (Weisz and Sinclair, 1987b). Nodule ureides in the 10 kPa O_2 treatment were approximately 130% of nodules in the 21 kPa O_2 treatment after 24 h (Table 3-2). For ureides to act osmotically in increasing nodule permeability for the 10 kPa O_2 treatment, it was tentatively concluded that they accumulated symplastically within the cortex. A symplastic accumulation of ureides would remove water from intercellular spaces and increase permeability.

The reason for ureide accumulation under the 10 kPa O_2 treatment was hypothesized to be a result of reduced ureide export. Ureide export from nodules depends upon loading of the xylem against a concentration gradient (Walsh et al., 1989b), which is presumably an energy dependent process. The vascular system in soybean is completely enclosed within an endodermis and three layers of pericycle

cells (Walsh et al., 1989b). Respiration and energy generation needed for xylem loading may be O_2 limited at a partial pressure of 10 kPa.

Ureides also accumulated in nodules after plants were exposed to a water deficit treatment for 7 h (Table 4-4). Accumulation of nodule ureides with water deficits was similar to ureide accumulation associated with treatments which decreased the amount of water supplied by the phloem (Walsh et al., 1989a). A decrease in phloem supply to the nodule decreases the amount of water available for export from the nodule through the xylem.

In the case of water deficit treatments, nodule permeability either decreased when nodules were exposed to a desiccating environment or remained constant when nodules were exposed to a non-desiccating environment (Figure 4-2). Water deficit treatments also caused a large decrease in $\bar{\Psi}_{tot}$ (Figure 4-4). A decrease in $\bar{\Psi}_{tot}$ would increase the hydrostatic tension applied to intercellular spaces, decrease the radius of pores remaining filled with water (equation 4-1), and increase permeability. One possibility for reconciling the observed response of decreasing or constant permeability under conditions of low $\bar{\Psi}_{tot}$ with the theoretical response of increasing permeability was that solutes accumulated in the apoplast. An increase in apoplastic solutes would decrease the hydrostatic tension applied to water in intercellular spaces, maintain water in intercellular spaces, and prevent an increase in nodule permeability.

The accumulation of ureides in response to water deficits (Table 4-4) may have served to decrease the hydrostatic tension if their accumulation was localized in the apoplast. Interruption of the phloem supply by nodule detachment also

caused a rapid decrease in permeability (Sung et al., 1991) which was associated with an increase in apoplastic ureides (Streeter, 1992).

The dependence on a continual phloem supply in maintaining nodule permeability and dinitrogenase has been established in stem girdling, stem chilling, and detopping experiments (Walsh et al., 1987). In these treatments, phloem supply was considered necessary to provide the nodule with sufficient soluble carbohydrates to support dinitrogen fixation. However, an alternative interpretation is that continual phloem supply was necessary to provide sufficient water for ureide export through the xylem.

In Chapter 5, a 20 mM EDTA solution with and without sucrose was forced through the secondary phloem at a stem girdle site, and these treatments were compared to girdled plants without a phloem source. Providing nodules with either an EDTA solution or sucrose and EDTA solution through the phloem maintained permeability at higher levels for the first 2 h following treatment than girdled plants without a phloem source (Figure 5-3). Providing nodules with the sucrose and EDTA solution through the phloem also resulted in higher rates of dinitrogenase activity after 2 h compared to providing the EDTA solution alone through the phloem (Figure 5-2). These results were consistent with the hypothesis that continual phloem supply was needed to export ureides through the xylem, and that soluble sugars in the phloem were necessary for the maintenance of dinitrogenase activity. Further development of the system described in Chapter 5 of providing nodules with carbohydrate and water through the exposed phloem tissue of a stem girdle offers potential in studying the effects of carbohydrate

source, ureide export from nodules, and growth regulators on dinitrogenase activity and nodule permeability.

An important finding of this work was that nodule permeability does not necessarily depend upon dinitrogenase activity. This was demonstrated in preliminary experiments on the effects of exogenous NH_3 on nodule permeability and dinitrogenase activity (Appendix). In response to 411 μL NH_3 L^{-1}, dinitrogenase activity decreased (Figure A-1) while nodule permeability was unaffected (Figure A-2). It was concluded that exogenous NH_3 does not have a controlling influence on nodule permeability. Similarly, exposing plants to water deficits while nodules were kept in non-desiccating conditions resulted in a large decrease in dinitrogenase activity (Figure 4-1) but had little effect on nodule permeability (Figure 4-2). This response demonstrates that nodule permeability is not dependent directly upon dinitrogenase activity.

A key question that remains to be determined is the location of solutes, and in particular the ureides, within the nodule in response to treatments affecting nodule permeability. The vacuum infiltration technique of nodules developed by Streeter (1992) has been valuable in demonstrating a time dependent increase in allantoate in the cortical apoplast of soybean nodules following nodule detachment. However, the vacuum infiltration technique is currently limited to detached nodules and small segments of nodulated roots.

An in vivo system to measure apoplastic/symplastic solute concentration has not been developed. However, if allantoate, or another charged ion in the nodule cortex, is responsible for water movement between the symplast and

apoplast then a microelectrode within an affected cell should measure changes in membrane polarization.

Pressure-probe measurements of cellular turgor pressures in the inner cortex would provide an indirect means of determining if cells in this region withdraw or release water from the apoplast in response to treatments affecting permeability. Additionally, pressure-probe measurements would allow determination of the elastic modulus at the cellular level. Conceivably, changes in the elastic modulus with conditions affecting permeability could determine water flux into and out of cells (equation 1-6).

Changes in nodule permeability are reversible (Weisz and Sinclair, 1987b). This suggests that if solutes are released to the apoplast to decrease nodule permeability, then there must also be a system to take solutes up from the apoplast to increase nodule permeability. Loading the cortex of nodules with labeled compounds under conditions of low permeability and then exposing nodules to 10 kPa O_2 (to increase nodule permeability) might result in an uptake of a particular solute from the nodule apoplast. The presence of a particular labeled solute in the xylem exudate would suggest an active uptake system.

This dissertation has been limited to studies of soybean, and data indicate that ureide accumulation, distribution, and transport may play a pivotal role in regulating nodule permeability. A final consideration is the mechanism of permeability regulation in legumes which do not produce ureides. Temperate legumes principally export asparagine and glutamine from nodules as the final products of dinitrogen fixation (Sprent, 1984). By analogy to the soybean model,

it is speculated that treatments which affect permeability may also affect the symplastic/apoplastic concentration of asparagine and glutamine.

A better understanding of permeability regulation in legumes may allow for increases in dinitrogen fixation. Nodule permeability and dinitrogen fixation in soybean are particularly sensitive to drought conditions. Drought results in a decrease in dinitrogen fixation prior to any effect on leaf gas exchange (Sall and Sinclair, 1991). Genetic differences occur in this response (Sall and Sinclair, 1991) which may be exploited in both understanding nodule permeability regulation and the development of cultivars capable of fixing dinitrogen under adverse environments.

APPENDIX
DINITROGENASE ACTIVITY AND NODULE GAS PERMEABILITY RESPONSE TO RHIZOSPHERIC NH_3 IN SOYBEAN

Introduction

The inability to increase dinitrogenase activity in legumes on a nodule weight basis through increased photosynthate availability (Finn and Brun, 1982; Walsh et al., 1987; Williams et al., 1982) has led researchers to explore other potential limitations to N_2 fixation. Recent work has indicated that dinitrogen fixation rates are closely linked quantitatively to nodule permeability (P) for O_2 diffusion (e.g. Hunt et al., 1987; Weisz and Sinclair, 1987b). Despite the observations of P adjustments in response to the environment (for recent reviews see Denison et al., 1988; Witty et al., 1990), there is a virtual void in understanding how P changes are achieved. Since NH_3 is the initial product of N_2 fixation, environmental factors which alter P and N_2 fixation should also affect NH_3 production in nodule bacteroids. Treatments which prevent NH_3 production such as prolonged exposure to 10% C_2H_2 (Minchin et al., 1983; Schuller et al., 1988) or replacement of the N_2:O_2 atmosphere surrounding nodules with an

Note: Portions of the material in this appendix were published in Plant Physiology (1990) 92:268-272. Used by permission.

Ar:O_2 atmosphere (Hunt et al., 1987; Minchin et al., 1983) decrease P. These reports have led to the suggestion that maintenance of steady state P is dependent upon the continual production of NH_3 (Minchin et al., 1983; Schuller et al., 1988). However, this hypothesis does not account for either the increase in P under conditions of low NH_3 production (as indicated by decreased ARA) associated with decreasing the rhizosphere pO_2 from 21 kPa to 10 kPa, or the decrease in P following an increase in rhizosphere pO_2 (Weisz et al., 1987b). A causal relationship between NH_3 production and P remains to be determined.

There have been no reports characterizing the effects of exogenous NH_3 on nodule permeability. The addition of NH_4^+ salts to the growth media of pea (Pisium sativium, L.) results in a decreased ARA of detached nodules (Houwaard, 1978; Houwaard 1980a), excised nodulated root systems (Bethlenfalvay et al., 1978), and intact plants (Houwaard 1978, 1980a) but has no short-term effect on the ARA of isolated bacteroids (Houwaard 1978, 1980b). In addition, NH_4^+ toxicity in plant tissues, may be overcome by increasing the light intensity (Criswell et al., 1976; Houwaard 1980b) or by the addition of organic acids to the culture media (Matsumoto et al., 1971). The objective of this study was to test the hypothesis that NH_3 regulates N_2 fixation through changes in nodule gas permeability, and that decreasing the availability of carbohydrates in the nodule would increase the sensitivity to NH_3 toxicity by decreasing the carbon skeletons needed for the initial assimilation of NH_3.

Materials and Methods

Plant Growth Conditions

Seeds of soybean (cv. Biloxi) were inoculated with a commercial preparation of Bradyrhizobium japonicum (The Nitragin Co., Milwaukee, WI) and germinated in an artificial soil medium (Terr-lite plug mix, WR Grace Co., Cambridge, MA). Three to four days after sowing, seedlings were removed from the soil mixture, the roots were rinsed with tap water, and the seedlings were transferred to a bored #3 stopper. The seedling and stopper combination was placed in the lid of a 1.5 L aeroponic chamber (Weisz and Sinclair, 1987a) containing half-strength, N-free Hoagland's solution (Hoagland and Arnon, 1938), and aerated with compressed air supplied to the bottom of each aeroponic chamber through an aquarium glass bead bubbler. Using this system, two plants per chamber were grown in the greenhouse under natural illumination with the photoperiod extended to 16 h by two incandescent light bulbs.

When plants had developed three to four fully expanded leaves they were transferred to the laboratory and placed under a combination of sodium and metal halide lamps on a 16-h photoperiod from 0500 to 2100 h. PPFD at the top of the plant was 2300 μmol m^{-2} s^{-1} for the high-light treatment or was reduced by a layer of shade cloth to 800 μmol m^{-2} s^{-1} for the low-light treatment. Plants were maintained under laboratory conditions from 2 to 4 days prior to treatment initiation. The aeroponic chambers in the laboratory were immersed in a water bath that maintained root and nodule temperature at 24°C. The air temperature around the shoots varied with room temperature and was approximately 22°C.

Treatment Implementation

The evening before an experiment began, the plant and stopper combination was transferred from the aeroponic chamber to an assay chamber as described by Weisz and Sinclair (1987a). The upper 9 cm of the nodulated root system of the intact plant was sealed into the assay chamber. The plant and assay chamber were placed on an aeroponic chamber so the lower root system was in continuously aerated nutrient solution.

The following morning (0730 EST) an initial measurement of dinitrogenase activity and nodule gas permeability was made using the "lag-phase" technique (Weisz and Sinclair, 1988a). The basis of this technique is that the time required to reach steady state C_2H_4 production rates following the introduction of a saturating concentration of C_2H_2 (Hardy et al., 1968) into a small assay chamber at a high volumetric flow rate is dependent upon nodule gas permeability (Weisz and Sinclair, 1988a). Experimentally, a 10% (v/v) C_2H_2 in air mixture was introduced into the assay chamber at a flow rate of 10 mL s^{-1} for a 2-minute period. One-mL gas samples were collected from the exit port of the assay chamber every 6 s for the 2 minute C_2H_2 exposure period. Acetylene was removed from the gas supply and the chamber was flushed with air for 1 minute before reducing the flow rate to 0.33 mL s^{-1}. The amount of C_2H_4 in the gas samples was determined by gas chromatographic procedures (Hardy et al., 1968).

Following the initial measurement of ARA and P, NH_3 was added to the humidified air passed through the assay chamber for an 8-h exposure period

beginning at 0800. ARA and P were periodically assayed during the NH_3 treatment and over a subsequent 15.5-h recovery period.

Known NH_3 concentrations were prepared by mixing anhydrous NH_3 with humidified air in a gas dosing device (Calibrated Instruments, Inc.). For both the high-light and low-light treatments, three plants were subjected to NH_3 concentrations of 0, 274, 411, 547, or 684 $\mu L\ L^{-1}$; an additional three plants were subjected to an NH_3 concentration of 821 $\mu L\ L^{-1}$ for the high-light treatment.

At the end of the experimental period, nodules were removed from the portion of the root system enclosed by the assay chamber, and nodule number, nodule fresh weight, and nodule dry weight were determined. Mean nodule diameter was estimated from a photocopy of the fresh nodules and used in the calculation of P (Weisz and Sinclair, 1988a).

Results and Discussion

Prevailing weather conditions and day length determined the irradiance on plants during their growth in the greenhouse. Plants used for the high-light treatment in the laboratory were grown in the greenhouse from 14 September 1988 to 20 December 1988, and plants used for the low-light treatment were grown from 10 December 1988 to 20 February 1989. The higher irradiance during the growth of the high-light treatment plants resulted in increased nodule dry weights and nodule numbers as compared to the low-light treatment plants (Table A-1). However, there was little difference between the initial ARA and initial P of nodules from plants exposed to the high or low-light treatment in the laboratory (Table A-1). These results agree with previous reports that increasing

Table A-1. Plant nodule characteristics, initial acetylene reduction activity (ARA), and initial nodule permeability (P) of NH_3 treatments. Values reported are the mean ± standard error of the mean (n=3 except where indicated).

Light	NH_3 Conc.	Nodule Dry Wt.	Nodule Number	Nodule Diameter	Initial† ARA	Initial P
	$\mu L\ L^{-1}$	----g-----		---mm---	$mm^3\ s^{-1}\ g^{-1}$	$\mu m\ s^{-1}$
High	0	0.32 ± 0.04	73 ± 16	2.6 ± 0.2	0.84 ± 0.01	19 ± 2
High	274	0.26 ± 0.03	42 ± 9	2.8 ± 0.2	0.89 ± 0.09	24 ± 4
High	411	0.22 ± 0.05	51 ± 13	2.5 ± 0.1	0.90 ± 0.17	20 ± 4
High	547	0.22 ± 0.07	44 ± 12	2.6 ± 0.1	0.85 ± 0.10	16 ± 2
High	684	0.21 ± 0.03	38 ± 4	2.7 ± 0.0	0.79 ± 0.05	20 ± 2
High	821	0.14 ± 0.03	32 ± 7	2.7 ± 0.1	1.26 ± 0.14	23 ± 3
‡Avg. of High Light		0.23 ± 0.02	47 ± 5	2.6 ± 0.1	0.92 ± 0.05	20 ± 1
Low	0	0.18 ± 0.02	34 ± 5	2.8 ± 0.1	1.15 ± 0.08	23 ± 2
Low	274	0.14 ± 0.02	23 ± 1	2.8 ± 0.2	1.15 ± 0.08	25 ± 1
Low	411	0.15 ± 0.02	31 ± 8	2.8 ± 0.2	0.94 ± 0.08	21 ± 3
Low	547	0.14 ± 0.01	31 ± 5	2.7 ± 0.1	1.18 ± 0.06	22 ± 2
Low	684	0.15 ± 0.01	38 ± 9	2.7 ± 0.2	1.06 ± 0.09	19 ± 2
§Avg. of Low Light		0.15 ± 0.01	32 ± 2	2.7 ± 0.01	1.07 ± 0.03	22 ± 1

† Units of ARA were expressed in $mm^3\ s^{-1}\ gdw^{-1}$ to be consistent with units of P. To convert $mm^3\ s^{-1}\ gdw^{-1}$ to $\mu mol\ h^{-1}\ gdw^{-1}$ (at 25°C) multiply by 147.

‡ n=18

§ n=15

photosynthate supply to nodules increases nodule mass but has little effect on specific dinitrogenase activity (Williams et al., 1982).

Dinitrogenase activity of the control treatment (0 μL NH_3 L^{-1}) was stable over the 24-h experimental period (Figure A-1) and confirms the absence of diurnal oscillations in dinitrogenase activity under conditions of constant rhizosphere temperature (Weisz and Sinclair, 1988b). Exposing nodules to an NH_3 concentration of 274 μL L^{-1} decreased ARA of the low-light treatment to approximately 70% of the pretreatment value while having no effect on ARA of the high-light treatment. At 411 μL NH_3 L^{-1}, ARA of both the low and high-light treatments decreased to around 60% of the pretreatment value, and at the end of the experiment, both light treatments had recovered to approximately 90% of the pretreatment value. An increase in the NH_3 concentration to 547 μL L^{-1} resulted in a further decrease in ARA but a difference existed between light treatments. When subjected to 547 μL NH_3 L^{-1}, ARA of the low-light treatment was decreased more, and showed less recovery, than ARA of the high-light treatment. Similarly, at 684 μL NH_3 L^{-1}, ARA of the low-light treatment was decreased more than the high-light treatment. However, neither the high-light nor the low-light treatment recovered ARA after 684 μL NH_3 L^{-1} was removed from the system. When nodules were exposed to 821 μL NH_3 L^{-1}, ARA was rapidly and completely inhibited and showed no signs of recovery following the NH_3 exposure period.

Nodule P was virtually unaffected at NH_3 concentrations below 547 μL L^{-1} (Figure A-2). At 547 μL NH_3 L^{-1}, P for both light treatments was reduced to 70

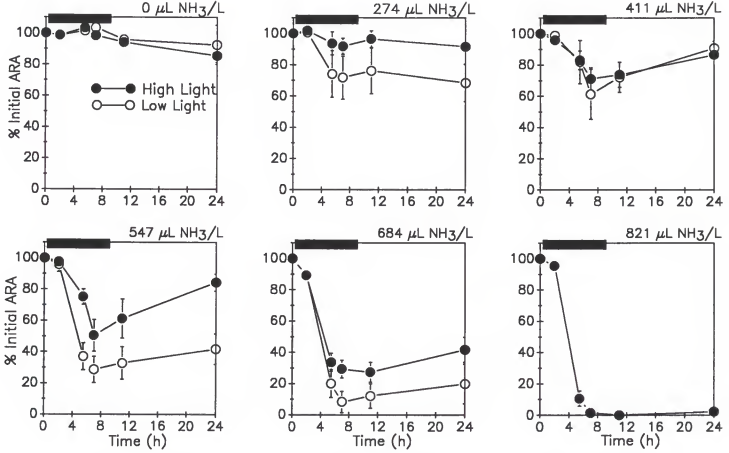

Figure A-1. Percent of the initial acetylene reduction activity (ARA) versus time for an 8 h NH_3 exposure (indicated by solid black bar). For absolute values of initial ARA, refer to Table A-1. Aeroponically grown plants were measured under high or low light conditions. Vertical bars represent ± standard error of the mean (n=3).

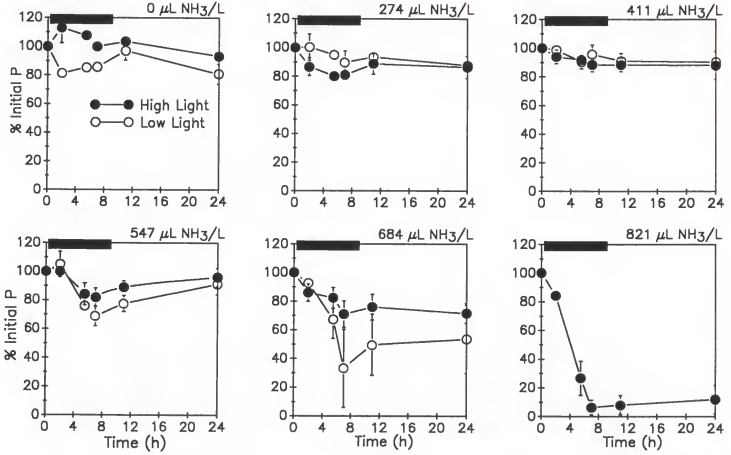

Figure A-2. Percent of the initial nodule permeability (P) versus time for an 8 h NH_3 exposure (indicated by solid black bar). For absolute values of initial P refer to Table A-1. Aeroponically grown plants were measured under high light or low light conditions. Vertical bars represent ± standard error of the mean (n=3).

to 80% of the pretreatment value and recovered to approximately 90% of the pretreatment value by the end of the experiment. At NH_3 concentrations of 684 and 821 $\mu L\ L^{-1}$, P rapidly declined during the first 5 h of NH_3 exposure. The determination of P by the "lag-phase" technique (Weisz and Sinclair, 1988a) depends upon measurement of time course changes in C_2H_4 production, and in some cases, ARA was undetectable after 7 h of NH_3 treatment at 684 and 821 $\mu L\ L^{-1}$ (Figure A-1). Under the conditions of rapidly decreasing ARA and P during the first 5 h of NH_3 exposure and 0 ARA thereafter, P was extrapolated to 0. This may have led to an underestimation of P and partially account for the large error bars at an NH_3 concentration of 684 $\mu L\ L^{-1}$ (Figure A-2). There was little recovery of P following the removal of NH_3 at concentrations of 684 or 821 $\mu L\ L^{-1}$.

A comparison of Figures A-1 and A-2 illustrates that the response of ARA to exogenous NH_3 was not tightly coupled to the response of P. A decrease in ARA was observed before there was any effect on P, and the relationship between ARA and P of the high-light versus the low-light treatment at 547 $\mu L\ NH_3\ L^{-1}$ indicates that recovery of P following NH_3 exposure was not associated with recovery of ARA. From these results it appears that although P is affected by NH_3, NH_3 does not exert a controlling influence over dinitrogenase activity by alterations in nodule permeability.

Excluding a direct effect of NH_3 on nodule permeability, exogenous NH_3 may decrease dinitrogenase activity in at least 3 other ways: (A) NH_3 could have a direct effect on dinitrogenase, on the ATP supply for dinitrogenase, or on the

electron transfer system to dinitrogenase (Laane et al., 1980). A direct effect of NH_3 on dinitrogenase activity is unlikely. Dinitrogenase is insensitive to changes in the NH_4^+ concentration (Dalton and Mortenson, 1972), and concentrations of up to 10 mM NH_4Cl have no effect on the dinitrogenase activity or the ATP/ADP ratio of pea bacteroids assayed in the presence of oxygenated myoglobin (Laane et al., 1980). (B) NH_3 assimilation requires carbon skeletons. Increasing the exogenous NH_3 concentration, and assimilating that NH_3, could deplete metabolic pools of organic acids normally available to supply energy equivalents and reductant for dinitrogenase activity. (C) Toxic concentrations of NH_3 may directly disrupt membrane systems (Puritch and Barker, 1967). A corollary of (B) and (C) is that maintaining an adequate supply of carbon skeletons needed for the assimilation of exogenously supplied NH_3 would prevent both the accumulation of toxic concentrations of NH_3 and membrane disruption.

An indirect effect of NH_3 on dinitrogenase activity as a result of decreased levels of energy and reductant would be indicated if dinitrogenase activity recovered after NH_3 was removed from the system and carbon skeletons were again available for respiratory production of ATP and NADPH. Such a response was observed after exposure of the high- and low-light treatments to 411 μL L^{-1} and after exposure of the high light treatment to 547 μL NH_3 L^{-1} (Figure A-1). The greater recovery of dinitrogenase activity after exposure to 547 μL NH_3 L^{-1} in the high-light treatment compared to the low-light treatment could be related to an increase in the carbohydrate pool size available for the assimilation of exogenous NH_3. Similar conclusions were drawn by Bethlenfalvay and Phillips

(1978) and Houwaard (1980) after finding that the inhibition of dinitrogenase activity in pea exposed to 8 mM $(NH_4)_2CO_3$ or 20 mM NH_4Cl, respectively, was counteracted by a doubling of the light intensity.

At NH_3 concentrations of 684 and 821 $\mu L\ L^{-1}$ there was little or no recovery of dinitrogenase activity after removal of NH_3 from the system (Figure A-1), and this was associated with an irreversible decrease in nodule permeability (Figure A-2). The ionization in tissue of NH_3 to form NH_4^+ would cause an increase in pH. Enzymatic activities, membrane potentials, and perhaps membrane permeability would be affected by changes in tissue pH. After nodules were exposed to 684 or 821 $\mu L\ NH_3\ L^{-1}$ they appeared water-soaked and may have suffered extensive membrane damage. In leaf tissue, application of NH_4^+ salts causes structural changes in chloroplasts and a loss of membrane integrity (Puritch and Barker, 1967). Similarly, a bacterial toxin inhibiting NH_3 assimilation causes leaf chlorosis as a consequence of NH_4^+ accumulating to concentrations as high as 61 mM (Turner 1981). At the reported pH of 6.2, this corresponds to an NH_3 concentration in the gas phase of 48.5 μM and is comparable, in this study, to the NH_3 concentrations suspected of causing membrane disruption (i.e. 684 $\mu L\ L^{-1} = 28\ \mu M$; 821 $\mu L\ L^{-1} = 34\ \mu M$).

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BIOGRAPHICAL SKETCH

Larry C. Purcell was born in Gainesville, Georgia in April of 1959. He grew up in the foothills of the Appalachian Mountains, fishing, hunting, and working on the family farm. After graduation from Gainesville High School, Larry attended the University of Georgia and received his Bachelor and Master of Science degrees in agronomy. After receiving his master's degree, he worked briefly as a county extension agent and then for 2 years as a research technician. Larry came to the University of Florida in January of 1988. After completion of his work at the University of Florida, Larry plans to continue his studies of dinitrogen fixation in a postdoctoral position at the University of Missouri.

Larry is married to Rebecca Zorn Purcell. They have one son, Jacob Zorn Purcell, and one dog, BJ.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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